

PHYTOCHROME PELLETABILITY, PHOTOTRANSFORMATION
AND DESTRUCTION IN MAIZE COLEOPTILES

by

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STATEMENT

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Noha Fuad.

Noha Fuad

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GENERAL DISCUSSION

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A Absorbancy

0.5PS Pellet resulting from a 500g centrifugation

0.5PS Supernatant " " " "

30KF Pellet " " " 30,000g "

30KE Supernatant " " " "

EDPA Ethylenediaminetetraacetic acid disodium salt

MOPS Morpholinopropane-sulfonic acid

SRB Resuspension buffer

SEBM Extraction buffer

SL Safelight

λ Wavelength

LIST OF ABBREVIATIONS

Pr	Form of phytochrome absorbing maximally in the red
Pfr	Form of phytochrome absorbing maximally in the far-red
R	Red
FR	Far-red
R/FR	Red followed by far-red
A	Absorbancy
0.5KP	Pellet resulting from a 500g centrifugation
0.5KS	Supernatant " " " "
30KP	Pellet " " " 30,000g "
30KS	Supernatant " " " " "
EDTA	Ethylenediaminetetraacetic acid disodium salt
MOPS	Morpholinopropanesulfonic acid
SRB	Resuspension buffer
SEBM	Extraction buffer
SL	Safelight
λ	Wavelength

SUMMARY

An action spectrum has been established for the induction, by short irradiation, of phytochrome pelletability in excised maize coleoptiles. It demonstrated that phytochrome is the primary photoreceptor for this response. It was also demonstrated that the induction of phytochrome pelletability in maize coleoptiles is a genuine phytochrome-mediated intracellular reaction.

Irradiations at wavelengths establishing a low photostationary level of Pfr were found to induce high levels of pelletability, comparable to those induced by red light. Red light was most effective in establishing high levels of pelletability under short irradiation conditions. Under continuous irradiation however, higher pelletability levels were obtained in the FR and blue regions.

Experiments were conducted using laser and flash-lamp pulse irradiations to prevent cycling of phytochrome between the Pr and Pfr states. These experiments were conducted to study relationships between phytochrome pelletability and phototransformation, and it was found that phytochrome could be photo-induced to become pelletable without necessarily going through the Pfr state. Several interpretations of the data are given which include the possibility that intermediates of phototransformation may be involved in the phenomenon of phytochrome pelletability.

It is also proposed that Pfr should not be regarded as a stable intermediate.

The maximal phototransformation achieved in a single flashlamp pulse was found to be increased if the same total light dose was given in two pulses separated by a dark interval. The curve obtained for this increase, as a function of the duration of the dark interval, revealed the existence of at least two intermediates of phototransformation which decay with half-lives of approximately 8ms and 26ms.

Phytochrome destruction in the dark following short irradiation, was analysed as a function of wavelength of irradiation, and temperature. Correlations established between phototransformation and rate and extent of destruction demonstrated the destruction of Pr as well as that of Pfr.

Phytochrome destruction appeared to correlate well with the initial level of pelletability attained following the inductive irradiation. It appears that the induction of phytochrome pelletability may indeed be a necessary step prior to the destruction of phytochrome.

It is concluded that the induction of phytochrome pelletability is a physiological response relevant to the control of at least one phytochrome-mediated response. It appears to reflect a close proximity existing *in vivo* between phytochrome and the partner X with which phytochrome co-pellets when tissues are subsequently fractionated. It is suggested that this close association has the capacity of resulting in an interaction of phytochrome with membranes.

It is also proposed that Pfr should not be regarded as the only active form of phytochrome.

During the course of this work, a number of difficulties associated with the determination of the levels of phytochrome pelletability and Pfr content have been demonstrated. Methods developed to rectify the problems encountered are described.

CHAPTER 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

FIG. 1.1

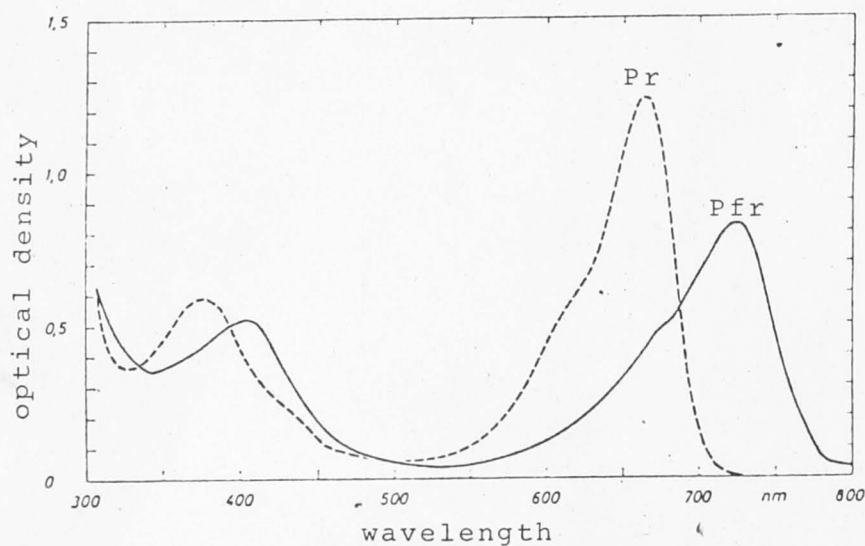


Fig. 1.1 Absorption spectra of purified oat phytochrome in the Pr and Pfr forms; Pr : direct measurements, Pfr : corrected for incomplete transformation of Pr to Pfr (taken from Hartmann 1966).

CHAPTER 1

Introductory background

Numerous developmental responses to light are mediated throughout the plant kingdom by a chromoprotein known as phytochrome (Parker and Borthwick 1950, Butler *et al.* 1959, Borthwick and Hendricks 1960, Mohr 1972; also Smith 1975, Satter and Galston 1976 for reviews). The responses range from the control of seed germination (Taylorson and Hendricks 1977) and photoperiodic responses like flowering (Vince-Prue 1975), to the control of chloroplast development (Mohr 1977), regulation of enzyme activities (Schopfer 1977) and control of trans-membrane ion fluxes (Satter and Galston 1976).

Phytochrome exists in two spectrally distinct isomeric forms (Siegelman and Firer 1964, Rüdiger 1972b). One form, called Pr, has a maximum of absorbancy in the red region near 660nm while the other form, Pfr, absorbs maximally in the far-red region near 730nm (see Fig. 1.1). These two forms are interconvertible by the absorption of light. Pr is phototransformed to Pfr most efficiently by the absorption of red light and conversely, Pfr to Pr by the absorption of far-red light (Butler *et al.* 1964a).

Due to the considerable overlap of their absorption spectra, particularly in the red region, the photoconversion of a population of phytochrome molecules into either form is never complete. For example, upon irradiation of phytochrome

by red light, molecules thus converted to Pfr are able to reabsorb red light and as a result are converted back to the Pr form. From this it follows that continuous irradiation of phytochrome with light of any wavelength produces a mixture of Pr and Pfr which eventually reaches equilibrium. The proportions of the total phytochrome pool present as Pfr and Pr at photoequilibrium are called the photostationary states for Pfr and Pr respectively:

$$[\text{Pfr}]_{\infty}^{\lambda}, [\text{Pr}]_{\infty}^{\lambda} \text{ (Butler et al. 1964a).}$$

The existence of a single pigment with these properties, thought to be present in plants in low concentrations, was predicted before the actual detection of phytochrome. The concept of a hypothetical photoreversible pigment was developed to rationalise the action spectra obtained for the photoperiodic control of flowering (Borthwick et al. 1948, Parker et al. 1946, 1950), the promotion of leaf expansion and of seed germination (Flint and McAlister 1935, Borthwick et al. 1952) and the inhibition of stem growth (Parker et al. 1949) by light. Red light was found to be most effective in inducing these responses, furthermore the induction of these responses by red light was reversible by far-red light; both the induction and its cancellation being repeatedly photoreversible. The prediction was fully verified by Butler et al. in 1959, who first detected the photoreversible pigment with a single beam spectrophotometer. The same authors devised a spectro-

photometric assay to detect phytochrome by means of dual wavelength absorption spectroscopy (Butler *et al.* 1959). The availability of a spectrophotometric assay based on the photoreversibility of phytochrome (see Chapter 3) led to its extraction and purification (see Rüdiger 1972a, Briggs *et al.* 1972, Kendrick and Smith 1976 for reviews) providing material for further physicochemical characterisation of the molecule.

The undegraded phytochrome chromoprotein is believed to have a molecular weight of 120,000 (Rice and Briggs 1973), with at least one covalently linked chromophore (Smith and Kendrick 1976, Spruit and Kendrick 1977, Scheer and Krauss 1977). The chromophore is believed to be a bilitriene (open chain tetrapyrrole with three methyne bridges) chemically related to that of phycocyanins (Rüdiger 1972b).

Although a lot is known about the physicochemical properties of phytochrome (Mumford and Jenner 1966, Burke *et al.* 1972, Tobin and Briggs 1973, Rice and Briggs 1973, Roux *et al.* 1975; see also Pratt 1978 for a review), the true chemical differences between Pr and Pfr are still unresolved (Rüdiger 1972b, Grombein *et al.* 1975a, Kendrick and Spruit 1977, Pratt 1978 for references).

The knowledge gained through the extensive application of the spectrophotometric assay and the studies of an increasing number of phytochrome-mediated physiological responses, may be summarised in the following scheme.

FIG. 1.2

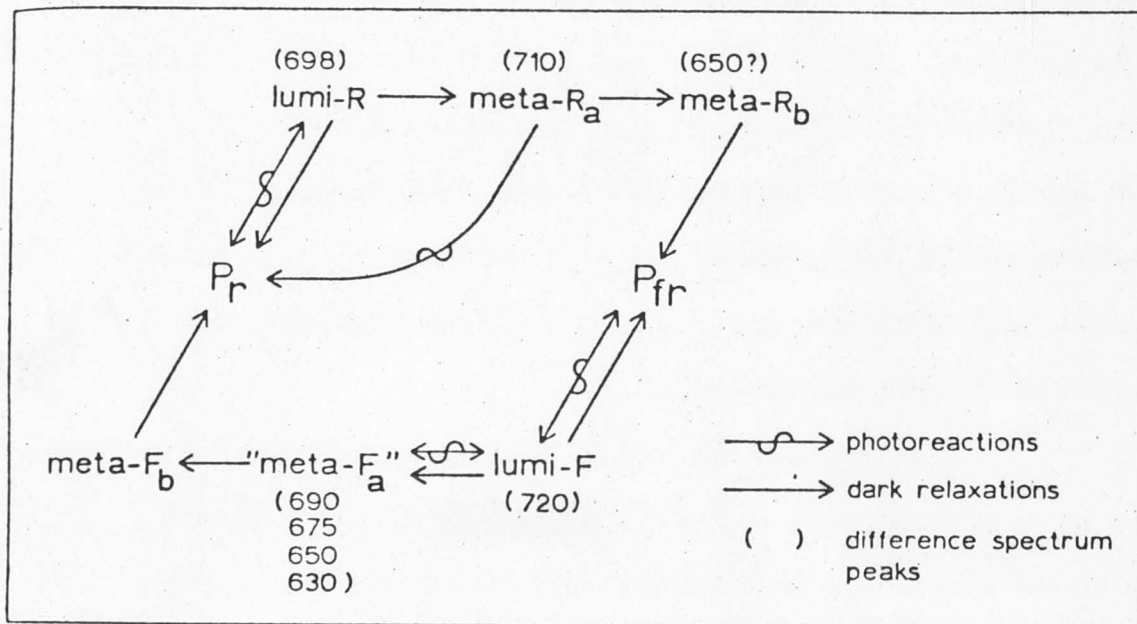
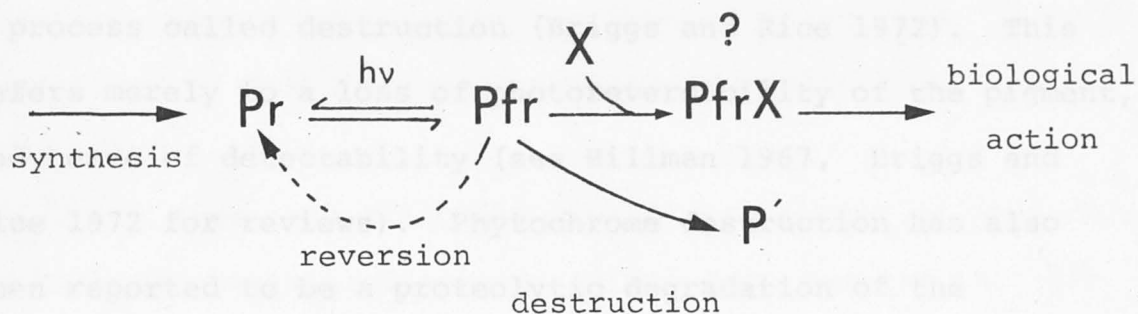


Fig. 1.2 Scheme for the phototransformation of phytochrome (taken from Kendrick and Spruit 1977).

Scheme 1(1)



Phytochrome is believed to be synthesised *de novo* (McArthur and Briggs 1970, Quail *et al.* 1973b, c, Coleman and Pratt 1974c), in the Pr form (Frankland 1972). Absorption of light converts it in a reversible manner to Pfr, which is believed to induce a biological response, possibly by association with a reaction partner X.

The course of phototransformation of phytochrome has been extensively studied by means of various spectroscopic techniques (Linschitz *et al.* 1966, Linschitz and Kasche 1967, Spruit 1966, Pratt and Butler 1968, Briggs and Fork 1969a, b, Kendrick and Spruit 1973a, b, Spruit and Kendrick 1973, Kendrick 1974). The phototransformation of phytochrome is now believed to be a sequential process. Each pathway of the phototransformation (Pr to Pfr and Pfr to Pr) consists of an initial photoreaction followed by a series of dark relaxations, and the two pathways do not appear to involve the same intermediates (see Kendrick and Spruit 1975, 1977 for reviews). Kendrick and Spruit (1977) have proposed the scheme reproduced in Fig. 1.2 to summarise these transformations.

Following irradiation, phytochrome disappears by a process called destruction (Briggs and Rice 1972). This refers merely to a loss of photoreversibility of the pigment, and hence of detectability (see Hillman 1967, Briggs and Rice 1972 for reviews). Phytochrome destruction has also been reported to be a proteolytic degradation of the chromoprotein (Quail *et al.* 1973b, Pratt *et al.* 1974).

In dicotyledons, Pfr may revert to Pr by a thermal process called reversion. It has been suggested that reversion and destruction do not compete for the same Pfr pool, and that reversion only concerns a small population of phytochrome (Schäfer and Schmidt 1974, Oelze-Karow and Mohr 1976). Reversion has not been reported to occur *in vivo* in monocotyledons (Hillman 1967), however it occurs in purified extracts from monocotyledons (Pike and Briggs 1972, see also Smith 1975 for references). Reversion is favoured by low pH and reducing agents, and is assumed to occur via a protonated form of Pfr (Mumford and Jenner 1971). It is proposed that the Pfr is relatively unstable and that under favourable conditions the requirement for far-red light to form Pr can be bypassed (Kendrick and Spruit 1973a). Inverse reversion, first thought to be the dark transformation of Pr to Pfr, has been observed in seeds (see Kendrick and Spruit 1977 for references). It is now recognised that this reaction is in fact the formation of Pfr in darkness, following FR irradiation. Pfr is believed to originate from the reversion

of an intermediate of phototransformation in the Pfr to Pr pathway, induced by FR light but blocked by extreme conditions of dehydration (Kendrick and Spruit 1974, Smith and Kendrick 1976).

Since the discovery of the involvement of phytochrome in photomorphogenic responses, it has been assumed that Pfr is the active form responsible for the numerous observed developmental events (Mohr 1962, 1966, Hendricks and Borthwick 1967, Smith 1970, Marmé 1974, 1977, Pratt 1978). As formulated in the above scheme: only Pfr is capable of instigating primary intracellular reactions which eventually lead to biological responses. This assumption is inferred from (i) the conventional experimental approach in which red (R) light (which produces Pfr) is used to induce a developmental response and far-red (FR) light to repress this induction (essentially removing Pfr), (see Hendricks and Borthwick 1967 for a review), and (ii) the close agreement between action spectra of various biological responses and those of the phototransformation reactions of phytochrome (Shropshire 1972).

Consequently, numerous investigators have attempted to correlate phytochrome content or state (Pr or Pfr) with the physiological response of etiolated tissues to red and far-red light. Hillman (1967) concluded that in any system examined with rigor, no rational analytically useful relationship could be established between a physiological response and

the state or content of spectrophotometrically assayed phytochrome. Growth responses in many tissues fail to correlate with spectrophotometric measurements of phytochrome state *in vivo* (Hillman 1967, see Kendrick and Hillman 1972 for a list of references).

Such situations have been called paradoxes by Hillman (1967). A notable example is the 'Zea paradox' in which the response (i.e. decrease of sensitivity of coleoptiles to blue light in phototropism) is induced maximally while no Pfr is detectable in the tissue (Briggs and Chon 1966). In the 'Pea paradox' (originally observed in peas by Hillman 1965, but also observed in *Phaseolus* by Klein *et al.* 1967, and in *Avena* by Hopkins 1971) the response induced by red light can be reversed by far-red light in the absence of detectable Pfr. The existence of these paradoxes has led to the concept of bulk and active phytochrome: bulk phytochrome is measurable but inactive, and in contrast the active form is not measurable by standard methods. Kendrick and Spruit (1972) warn however that the problems associated with these paradoxes may not be real but only problems of the *in vivo* spectrophotometric assay in which whole pieces of plant tissue are used.

There are instances where apparent direct correlations between responses and Pfr levels have been reported. In most cases however, the correlations were based on estimated or extrapolated Pfr contents rather than on actual spectro-

photometric measurements (Drumm and Mohr 1974, Morgan and Smith 1976), while Oelze-Karow and Mohr (1973) have used the lipoxygenase assay to determine Pfr levels *in vivo* and found very good correlation with the spectroscopic determination of Hartmann and Spruit (1968, in Hanke *et al.* 1969).

In addition to the R/FR photoresponses described above, phytochrome is thought to mediate responses to irradianations of long duration (high irradiance responses, HIR). Under prolonged irradiation, maximum action usually occurs in the FR (near 720nm) and the blue regions, where neither Pr nor Pfr absorb maximally. An important characteristic of these responses is a strong irradiance dependancy of the light reaction. Several hypotheses have been advanced to explain HIR on the basis of phytochrome only. The alternative is to attribute HIR to the function of one or even several photo-reactive systems besides phytochrome, i.e. a blue light receptor to account for the peak of action in the blue. Hartmann (1966, 1967) has obtained convincing experimental evidence that the far-red peak of action is due in some ways to the phytochrome system. It has been proposed that phytochrome may act via an excited form of Pfr (Schopfer & Mohr 1972, Mohr 1972), or that newly formed Pfr may be more active than old Pfr (Hartmann and Unser (1973). Two model theories have been proposed to explain HIR. Schäfer (1975c) proposed an open phytochrome-receptor model in which a short-lived transient

state of Pfr (PfrX) is the effector molecule. This theoretical model predicts both action in the blue and far-red regions. Deutch and Deutch (1974) calculated a kinetic model based upon a cyclical phytochrome conversion scheme to explain barley leaf unfolding to different irradiances of far-red light. In this model, they proposed that two physiologically active transitory forms of phytochrome (different from Pfr) are produced.

The central problem of photomorphogenesis is to explain how the active form of phytochrome interferes with the cellular metabolism of the plant to trigger the series of reactions resulting in a photomorphogenic effect.

Attempts to rationalise the diversity of phytochrome-mediated responses have resulted in the formulation of two major hypotheses: the 'gene' and the 'membrane' hypotheses.

1) Mohr (1966) proposed that phytochrome controls plant development by regulating gene activation and repression. Positive evidence for involvement of the genome has come from (i) experiments showing increase in RNA and protein content under the regulation of phytochrome, and (ii) the demonstration that transcriptional and translational inhibitors can block the expression of a number of Pfr induced responses (Mohr 1966). This gene regulation hypothesis predicts a specific regulation of mRNA and consequently of enzyme protein synthesis. As a result, tests to show to what extent this hypothesis may be

valid, have involved many attempts to demonstrate the regulation by Pfr of *de novo* synthesis of enzymes, which play a central role in photomorphogenesis. In many cases, the results remain controversial (Schopfer 1977, 1972).

2) Hendricks and Borthwick (1967) proposed that phytochrome may act by altering membrane permeability. This theory was developed in recognition of the fact that phytochrome-mediated responses are known which are not only too rapid to be explained in terms of gene regulation, but in some cases at least, have also proved insensitive to transcriptional and translational inhibitors (Satter and Galston 1973). For example, the nyctinastic leaflet movement in *Mimosa pudica* can be detected within 5 min following the light treatment (Fondeville *et al.* 1966). A similar rate of display has been found with leaflets of *Albizzia julibrissin* and other legumes (Hillman and Koukkari 1967, Jaffe and Galston 1967). Leaflet movements are now thought to be caused by massive osmotic changes in the motor cells brought about by changes in membrane permeability (Satter *et al.* 1970, Satter and Galston 1971, Schrempf *et al.* 1976). Studies of other fast responses supporting this hypothesis have been reviewed by Satter and Galston (1976) and Marmé (1977).

In the later development of these hypotheses, it is often postulated that a receptor X (or reaction partner) exists for Pfr, to which Pfr must bind to exert its action

(see scheme 1(1)). This postulated receptor need not be unique and may be different in the induction of various reactions by Pfr (Mohr 1972).

In the 'membrane' hypothesis, the most favoured candidate for the postulated receptor (see scheme 1(1)) is either a membrane or an intrinsic component of a membrane. While Hendricks and Borthwick (1967) pointed out that the actions of Pfr affecting membrane permeability do not necessarily require the presence of phytochrome in a membrane, for many years investigators have searched for an association of phytochrome at least in part with cellular membranes, to test the 'membrane hypothesis'. Support for such an association comes from studies on the subcellular localisation of phytochrome.

The association of phytochrome with a membrane structure, possibly the plasmalemma (Haupt 1972b), was inferred from results of physiological experiments with polarised light in *Dryopteris* (Etzold 1965), in *Mougeotia* (Haupt 1970) and in maize coleoptiles (Marmé and Schäfer 1972).

Galston (1968) attempted to localise phytochrome *in situ* by microspectrophotometry. He was able to scan different regions of cells from oats and peas with a micro-beam, and record the absorbance spectra of the region scanned. He detected small reversible absorbance changes in the nuclear region, reminiscent of those of phytochrome, following red and far-red irradiations. From these results Galston concluded

that there was evidence for the presence of phytochrome at or near the nuclear membrane (Galston 1968).

Pratt and co-authors developed and used an immunocytochemical technique to localise phytochrome *in situ*. Their studies showed that stain for phytochrome was usually observed distributed throughout the cytoplasm, while stain for phytochrome could also be observed in association with amyloplasts and mitochondria, and in some tissues with the nuclear membrane (Pratt and Coleman 1971, Coleman and Pratt 1974a, b).

Attempts to ascertain the subcellular distribution of phytochrome have also been made in cell fractionation studies.

Two approaches can be operationally described.

(1) In the first approach, the localisation of phytochrome involves the search for phytochrome-mediated responses in purified organelles or membrane fractions. This work has been recently reviewed by Marmé (1977) who concluded that the data support the hypothesis that phytochrome (at least in part) is located in various membranes.

(2) The alternative is to attempt to observe *in vitro*, a naturally occurring association between phytochrome and subcellular fractions. This approach is based on the spectrophotometric detection of phytochrome in the various subcellular fractions resulting from cell fractionation.

Attempts to identify the phytochrome containing components

mainly involve correlation in the distribution profiles of the pigment and putative membrane or organelles markers.

Phytochrome pelletability

The first evidence for the possible association of phytochrome with subcellular particulate material was obtained by differential centrifugation of homogenates extracted from etiolated tissues (Gordon 1961). Eight years later Rubinstein *et al.* (1969) reported that a small fraction of cellular phytochrome could be pelleted at 40,000g from homogenates of oat seedlings, and that this fraction might be in some way associated with cell membranes.

It is now well documented that two kinds of association of phytochrome with particulate material exist.

(1) A small proportion (5-8%) of the total cellular phytochrome of dark grown plants is found inherently associated with particulate material (Rubinstein *et al.* 1969, Quail *et al.* 1973a). This inherent association has not been well studied. There is some evidence however that this small proportion corresponds to that phytochrome which has been found in purified organelles via the first approach above (e.g. mitochondria: Manabe and Furuya 1974, Furuya and Manabe 1975; etioplasts: Cooke *et al.* 1975).

(2) A larger (up to 80%) proportion of the total phytochrome pool may be photoinduced to become associated with particulate material (hence pelletable). This red light-enhanced phytochrome pelletability can be induced by irradiating

intact plant tissues (*in vivo*) or cell-free homogenates derived from them (*in vitro*). The pelletability induced by irradiation of extracts at 0°C has only been observed in those from *Cucurbita* (Marmé *et al.* 1973, Marmé 1974), *Sinapis* (Pratt and Marmé 1976), peas (Yamamoto and Furuya 1975). It has been extensively studied only in *Cucurbita* (Quail 1975a, b, c, Quail and Gressel 1975) and found to be dependent upon low ionic strength and pH. It can occur in the absence of added divalent cations. Pratt in his review (1978) on the *in vitro* pelletability of *Cucurbita* supports the interpretation of Quail that in this material 'it is almost certain that it represents an artifactual electrostatic interaction between phytochrome and ribonucleoproteins [such an association] does not occur *in situ* and cannot be implicated in the mode of action of phytochrome'.

In contrast to the induction of pelletability *in vitro*, the *in vivo* induction has been observed in all material studied so far (Pratt and Marmé 1976). The resultant pelletability of phytochrome is very much dependent on added divalent cations in the extraction medium, while it is relatively insensitive to pH and ionic strength. Absence of divalent cations in the extraction medium (Mg^{++} is generally used) reduces the pelletability of phytochrome from red irradiated tissue to an amount similar to that of unirradiated controls. It is a matter of debate whether Mg^{++} preserves *in vitro*, a pre-existing intracellular association, or whether

it induces the '*in vivo* potentiated' association during homogenisation of the tissue. Yu and co-workers (Yu 1975a, b, c, Yu and Carter 1976c, Yu *et al.* 1976) attempted to answer this problem by pretreating red irradiated material with glutaraldehyde before homogenisation of the tissue in Mg^{++} -free buffer. Glutaraldehyde was used as a cross-linking reagent to stabilise any association between phytochrome and subcellular constituents prior to extraction. Enhancement of phytochrome pelletability by red light was also demonstrated using this procedure; such treatments resulted in yields of pelletable phytochrome similar to those obtained by extraction with Mg^{++} -containing buffer (Yu 1975a, b). Yu and Carter (1976c) have interpreted these results to constitute evidence in favour of the hypothesis that irradiated phytochrome does interact intracellularly with a fraction which becomes particulate upon homogenisation. This particulate partner has not been identified yet, as the distribution profiles of pelleted phytochrome on sucrose density gradients fail to correlate with those of markers for mitochondria, endoplasmic reticulum, plastids or plasma membrane (Marmé 1974, Quail *et al.* 1976, Yu 1975c, Yu *et al.* 1976).

It is now known that the *in vivo* red light-induced phytochrome pelletability is a very fast response going to completion within 5-10 sec at 22°C in oats. It can be reversed at 0°C if the inducing red irradiation is very short and is followed very rapidly by far-red light (Pratt and

Marmé 1976). If longer irradiations are used to induce pelletability (3-5 min), far-red light has no immediate effect in reducing pelletability.

Although pelletability once induced *in vivo* by 3-5 minutes of red light is not reversible by far-red light, it is slowly decreased during subsequent incubation of R/FR irradiated tissues in darkness. This decrease is temperature dependent and occurs at different rates in different material (Quail *et al.* 1973a, Boisard *et al.* 1974, Yu 1975b, Pratt and Marmé 1976).

It is interesting to note that subcellular distribution of phytochrome, studied via cell fractionation and the spectrophotometric assay, have been paralleled by immunological localisation of phytochrome *in situ* after red light treatment. Using their indirect phytochrome labelling method, Mackenzie *et al.* (1975) and Pratt *et al.* (1975) found that in oats and rice, phytochrome is generally distributed throughout the cytoplasm in cells of seedlings that had not been exposed to light prior to fixation. However, within five minutes from the onset of saturating red irradiation, phytochrome (as Pfr) becomes associated with discrete regions of the cell. After transformation back to the red absorbing form originally present, phytochrome gradually resumes its original general distribution.

Scope of the work

In a sequence of events starting from the light absorption by phytochrome and ending in the observation of

the resulting morphogenic response, it is evident that a study of the reactions the least remote in time from the primary excitation of the pigment may result in a better understanding of the primary mode of action of phytochrome. The type of phytochrome-mediated responses studied has included very short term phenomena such as the control of flowering in *Pharbitis* and *Kalanchoe* (Nakayama *et al.* 1960, Fredericq 1964), the potentiation of epinasty in wheat (Wagné 1965), the control of the Shibata shift by phytochrome (Mohr 1977), changes in bioelectric potentials (Tanada 1968, Jaffe 1968, Newman and Briggs 1972), and changes in permeability to water (e.g. Weisenseel and Smeibidl 1973). This has brought phytochrome research closer and closer to the initial event of light perception by the plant.

The *in vivo* induction of phytochrome pelletability by red light is an important reaction to investigate for at least two reasons: (i) since it is now known to be one of the fastest responses to red light observed so far, it has the potential of representing one of the primary reactions involved in phytochrome mode of action, and (ii) it also has the potential of testing the hypothesis that a receptor for Pfr exists, and if so to identify it. This is of primary importance in the formulation of hypotheses concerning phytochrome modes of action.

Before any such hypotheses can be advanced on the basis of studies of the light induction of phytochrome

pelletability, a number of uncertainties regarding this reaction must be clarified however.

(1) At the time of starting the present study, there was no evidence as to whether or not the red light-enhanced phytochrome pelletability is a phytochrome-mediated response. R/FR reversibility had not been demonstrated. Instead, red light-enhanced pelletability was found to be partially reversed by far-red light in pumpkin, while in the case of maize, R/FR (i.e. red irradiation followed by far-red irradiation) increased the level of pelletability as compared to red alone (Quail *et al.* 1973a). This observation also raised the question of the overall specificity of the phenomenon (Quail 1975b).

(2) In the event that phytochrome pelletability could be shown to be a phytochrome-mediated response, the controversial nature of the phytochrome-particle association observed must be clarified. It is important to establish whether or not Mg^{++} induces the association of phytochrome with the particulate partner *in vitro*, at any step during fractionation of the irradiated tissue, and whether or not the association found *in vitro* reflects an *in vivo* situation. This is of prime importance if it is to be shown that a receptor for Pfr exists and to identify the nature of the postulated receptor.

(3) There is as yet no evidence to show that the *in vivo* red light-induced phytochrome pelletability is related to

phytochrome action. For instance, if it can be established that as a result of irradiation, phytochrome binds to a receptor X, the question arises as to whether or not this binding is a necessary step in the series of reactions that lead to biological display.

(4) The establishment of theories on the mode of action of phytochrome also relies on the knowledge of the active form of phytochrome. As already pointed out, a controversy which arises from both the existence of the phytochrome 'paradoxes' and the rationalisation of HIR, exists even regarding this fundamental point. The involvement of other forms than Pfr in phytochrome action may need to be considered.

It is the intention of the present study to investigate the *in vivo* light induction of phytochrome pelletability in maize coleoptiles, with the hope of clarifying the various uncertainties described. Phytochrome pelletability will be related to phototransformation and destruction of phytochrome in an attempt to answer the last two questions.

I have chosen maize coleoptiles as a working material because:

(1) Phytochrome pelletability has not been shown to be induced *in vitro*, at least at 0°C in this material (Marmé 1974, Marmé *et al.* 1975), and no association with 31S degraded ribosomal material has been observed in maize (Quail 1975b). Hence it is hoped that the study of the *in vivo*

induction of phytochrome pelletability in maize should not be interfered with by the apparently artifactual *in vitro* association of phytochrome with particulate material reported in *Cucurbita* (see above).

(2) The requirement for Mg^{++} in the extraction buffer for the expression of the light enhanced pelletability has been successfully obviated in maize coleoptiles by the treatment of the irradiated tissue with cross-linking reagents (Yu 1975a, b, c, Yu and Carter 1976c, Yu *et al.* 1976), hence it is hoped that useful comparisons between the two experimental approaches may be established.

(3) Maize is a monocotyledon, thus problems associated with the dark reversion of Pfr should not be encountered (see above), hence reducing the complexity of the system.

(4) The maize coleoptile is a suitable experimental material for this study because a number of phytochrome-mediated responses have been characterised in this system (Briggs 1963, Chon and Briggs 1966, Wilkins and Goldsmith 1964, Duke and Wickliff 1969, Duke *et al.* 1977). The coleoptile is also known to be a good source of phytochrome (Briggs and Siegelman 1965), and last, it is an organ easily harvested free of primary leaves, and easy to handle in the dark.

The disadvantages in this choice are that no detailed molecular characterisation of maize phytochrome has been achieved so far, immunochemical assays have not been developed for phyto-

chrome and maize seedlings are known to be a difficult material to grow uniformly.

Following a description in Chapter 2 of the material and methods used in this study, Chapter 3 will be an exposé of methodological problems encountered in the measurements of Pfr content and determinations of phytochrome pelletability, together with their rectification. The involvement of phytochrome in mediating its own light induced pelletability will be dealt with in Chapter 4, while the possible involvement of other forms of phytochrome than Pfr in its pelletability and destruction will be the subject of Chapters 5 and 6 respectively. Chapter 6 is also concerned with the relevance of phytochrome pelletability in the destruction reaction. Finally, Chapter 7 will attempt to finalise the answers to the questions raised here by discussing relevant data, obtained in the various chapters, in relation to each other and in the light of other studies reported in the literature. From there, views on phytochrome mode of action will be expressed.

CHAPTER 2

GENERAL MATERIAL AND METHODS

CHAPTER 2

This chapter describes the material and the methods routinely used in the experiments presented in this thesis. Details of particular techniques and methods used in specific cases will be dealt with separately in the relevant chapter.

Plant material

Dark grown maize seedlings were used throughout this study. Maize (*Zea mays* L.C.V. GH390) was supplied by:

a) Rewlee Pty.Ltd. Bodalla, NSW.

Rewlee Top Crop (1976).

b) H.J. & A.B. Dorsman, Singleton, NSW.

HY Yield (1978).

The seeds were supplied coated with a fungicide-insecticide mixture containing 75% Thiram, 10% Malathion, 5% Lindane. Seeds were washed and soaked in running tap water for 24 hours prior to sowing. For consistency, soaking was initiated and sowing carried out regularly at about 5pm daily. Approximately 300 soaked seeds were distributed evenly in plastic trays (31 x 26 x 12 cm) over 4 layers of paper towels (Kleenex), moistened with 250ml of distilled water. The trays containing the seeds were covered with a plastic lid and a black cloth and then enclosed in light-proof cardboard boxes. The boxes were

placed in a temperature controlled room at 30°C.

Coleoptiles free of primary leaves were excised from the etiolated seedlings obtained 4³/₄ - 5 days after sowing. The apical section of 1 - 2cm constituted the starting material for all experiments.

Safelights

All handling of dark grown seedlings was performed in dark rooms equipped with dim green safelights. The three types of safelights described below have been used. They represent consecutive replacements corresponding to gradual improvements of their spectral characteristics as safelights, as will be discussed in Chapter 3.

- a) Type A was obtained by filtering light from a daylight fluorescent tube (OSRAM 40W) through a layer of amber (Rohm 303) and a layer of blue (Rohm 627) plexiglass.
- b) In type B, green fluorescent tubes (Philips TL 40W/17) replaced the daylight tubes of type A.
- c) In type C safelights, a far-red absorbing filter was used in conjunction with the combination of Rohm filters described above (type C1) or with a combination of Cinemoid filters (Deep Blue No. 20, Yellow No. 1 and Chrome Yellow No. 46) (type C2), to filter the light from the green fluorescent tubes. The far-red absorbing filter consisted of a 1 cm

FIG. 2.1

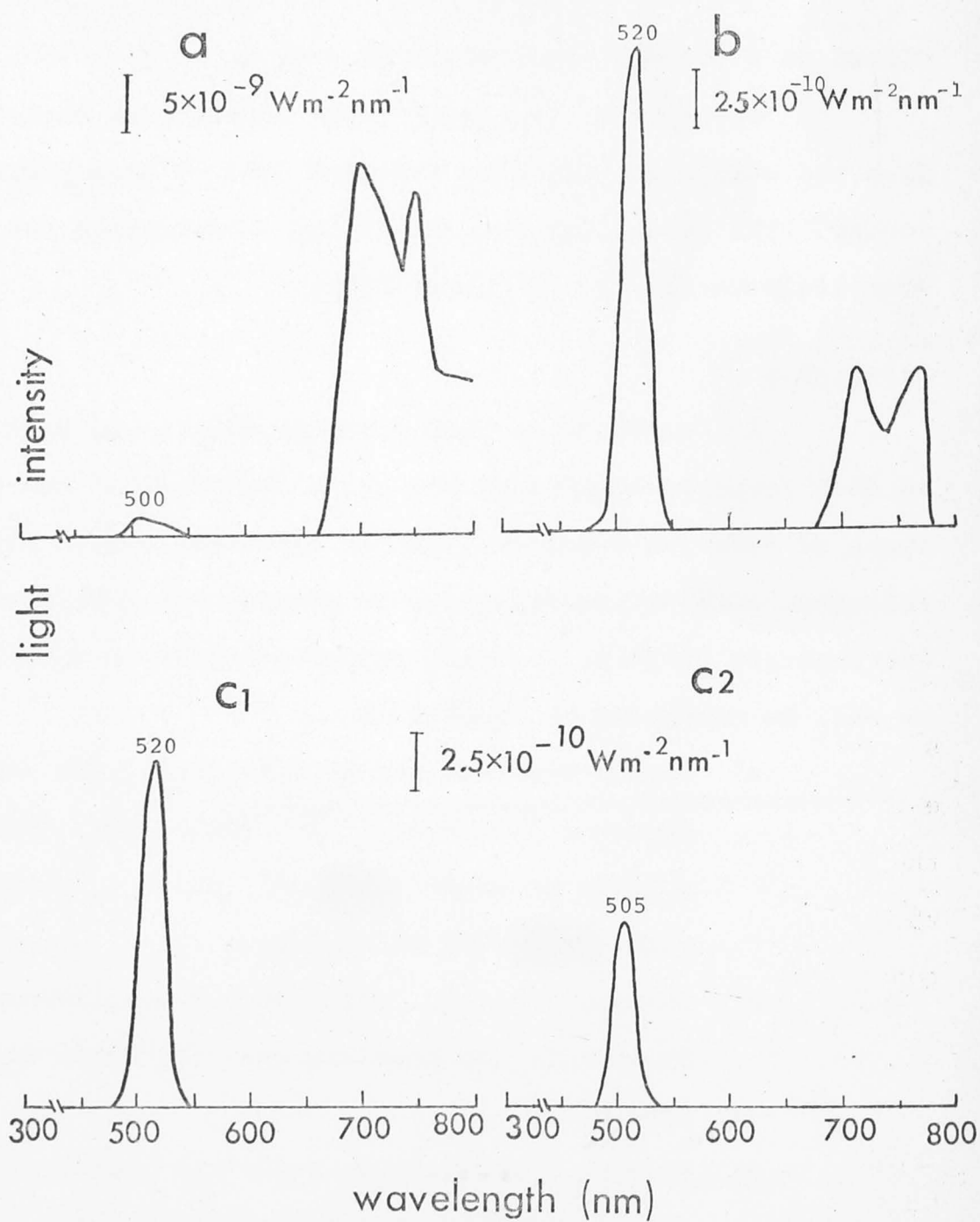


Fig. 2.1 Spectral distribution of the various safelights; a, b, c1, c2: safelights type A, B, C1 and C2 respectively, as described in the text.

deep solution of cupric sulphate (100g/l)
and sulphuric acid (0.2% vol. concentrated
 H_2SO_4).

The spectral intensity distributions of the different safelights from 800nm to 300nm was analysed using an IL780 spectroradiometer and is shown in Fig. 2.1.

The figure shows that in type A safelights, the light emission in the green region (max. at 500nm) is small compared with that in the far-red region ($> 680\text{nm}$). The use of green light fluorescent tubes, in type B safelights enhanced the green emission component relative to the far-red component: the far-red emission was nevertheless substantial and was found to be mainly produced near the extremities of the fluorescent tubes. Further reduction of the far-red component was therefore achieved by covering these ends with opaque tape or black cloth (i.e. type B construction). The spectral distribution of type B safelights is shown in Fig. 2.1b. The green (max. at 520nm) component is greatly enhanced relative to the far-red component. In type C safelights emission of light is strictly limited to the green region only, with emission peaks at 505nm and 520nm for types C2, C1 respectively.

The light intensity at bench level ($\sim 1\text{m}$ from light) was 0.2 Wm^{-2} for type A, 1 mWm^{-2} for type B and 0.4 mWm^{-2} and 0.2 mWm^{-2} for types C1 and C2 respectively.

TABLE 2.1
Schott interference filters used in conjunction with
the M3 monochromator

Type B and C safelights did not cause detectable phototransformation of Pr to Pfr nor did they induce phytochrome pelletability, compared to dark controls, even upon prolonged exposure (up to 5 h) of excised maize coleoptiles (see Fig. 4.5).

Monochromator and filters

For irradiation of plant tissues, cell-free homogenates and subcellular fractions derived from them, monochromatic light was obtained using Schott interference filters in conjunction with an M3 monochromator (Heinze Zeuschel, Tübingen) and a 150W light source (Philips, 6423). The beam divergence through the interference filters was less than 10 degrees, which is well within the tolerance limits of the filters.

All filters used were checked for their spectral characteristics from 800nm to 300nm with a Varian series spectrophotometer (model 6348) or a Zeiss spectrophotometer (Carl Zeiss, M4 QIII & PMQII, Germany).

Table 2.1 lists the filters used with their spectral characteristics.

Light intensity obtained from the monochromator through the filters was checked periodically for consistency using a YSI-Kettering Model 65A radiometer.

Red (R) and far-red (FR) irradianations usually refer to irradianations with monochromatic light obtained through DEPIL/DIL interference filters with maximum of transmission between 658nm and 663nm, and 727nm or 729nm respectively.

Schott interference filters used in conjunction with the M3 monochromator.

λ max nm	Type	T max %	H.W. nm
425	DIL	12.5	9
447	DEPIL	13	10
473	AL	40	20
509	DEPIL	24.5	10
532	DEPIL	23	10
538	DEPIL	14	11
552	DEPIL	23	10
578	DEPIL	19	9.5
601	DEPIL	18	8
621	DEPIL	15	9
658	DEPIL	18.5	11.2
661	DEPIL	16	10
663	DIL	20	10
675	DEPIL	18	9
692	DEPIL	17	12
706	DEPIL	17	9
717	DEPIL	9	10.5
721	DEPIL	11.7	11
727	DEPIL	15	11
729	DEPIL	15	9.5

λ max: wavelength at the maximum of transmission

T max: maximum transmission

H.W. : bandwidth at half maximum transmission

DEPIL, DIL: line, double interference filter

AL : band, single interference filter

DEPIL, DIL filters did not have detectable secondary transmission peaks

AL filter 473 was used in conjunction with a Kodak wratten filter No. 44.

For irradiation, coleoptiles were so arranged in a petri dish that they did not overlap each other. Irradiations were usually carried out at room temperature.

Ratiospect

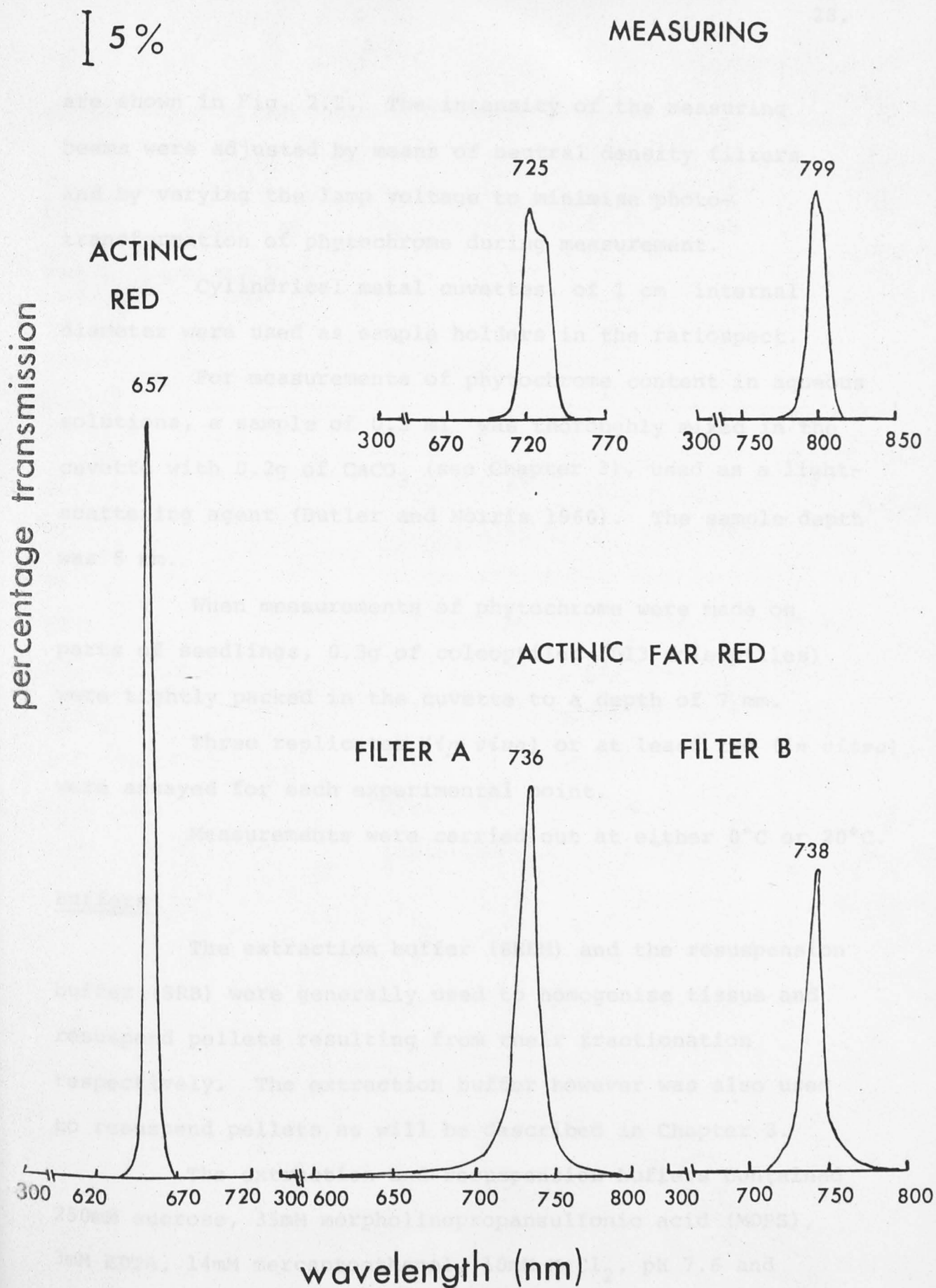
Phytochrome measurements were made on subcellular fractions (*in vitro*) or parts of seedlings (*in vivo*) in a dual wavelength spectrophotometer (Ratiospect model R-2, Agricultural Specialty Co., Hyattsville, Maryland) equipped with an automatic timing system according to the design of Marmé (1969). The automatic timing device provides a cycle of successive red and far-red actinic irradiations of 30 sec duration each, separated by 30 sec intervals for absorbancy measurements. Duration of irradiation and measurements could also be controlled manually.

Actinic (657nm and 737nm) and measuring beams (725nm and 799nm) were obtained from DFA (150W) and DEF (150W) bulbs (General Electric). The light was collimated through Schott interference and cut-off colour filters. The suitability of the filters used to produce the red and far-red actinic irradiations will be discussed in Chapter 3. It suffices to say here that the cut-off filter used initially in combination with the 737nm interference filter to remove transmission in the blue region, was replaced by another with a cut-off at a longer wavelength. These two filter combinations will be referred to as filter A and filter B respectively in the text and figures. The transmission spectra of the filters and filter combinations used

Fig. 2.2 Transmission spectra of the interference filters used in the Ratiospect to produce measuring and actinic irradiances; actinic far-red: combinations of interference and cut off filters. The spectra were taken in a Varian series spectrophotometer (model 6348).

percentage transmission

FIG. 2.2



are shown in Fig. 2.2. The intensity of the measuring beams were adjusted by means of neutral density filters and by varying the lamp voltage to minimise photo-transformation of phytochrome during measurement.

Cylindrical metal cuvettes, of 1 cm internal diameter were used as sample holders in the ratiospect.

For measurements of phytochrome content in aqueous solutions, a sample of 0.3 ml was thoroughly mixed in the cuvette with 0.2g of CaCO_3 (see Chapter 3), used as a light-scattering agent (Butler and Norris 1960). The sample depth was 5 mm.

When measurements of phytochrome were made on parts of seedlings, 0.3g of coleoptiles (13 coleoptiles) were tightly packed in the cuvette to a depth of 7 mm.

Three replicates (*in vivo*) or at least two (*in vitro*) were assayed for each experimental point.

Measurements were carried out at either 0°C or 20°C.

Buffers

The extraction buffer (SEBM) and the resuspension buffer (SRB) were generally used to homogenise tissue and resuspend pellets resulting from their fractionation respectively. The extraction buffer however was also used to resuspend pellets as will be described in Chapter 3.

The extraction and resuspension buffers contained 250mM sucrose, 35mM morpholinopropansulfonic acid (MOPS), 3mM EDTA, 14mM mercaptoethanol, 10mM MgCl_2 , pH 7.6 and 250mM sucrose, 25mM MOPS, 3mM EDTA, 14mM mercaptoethanol,

pH 7.1 respectively, unless otherwise stated.

Chemicals

A listing of the chemicals used with their origin is given in Table 2.2.

TABLE 2.2

CHEMICALS USED

Chemical	Source
Sucrose	Univar-AJAX-Sydney
MgCl ₂	Univar-AJAX-Sydney-Melbourne
CaCO ₃	Univar-AJAX-Sydney-Melbourne
EDTA	Univar-AJAX-Sydney-Melbourne-Perth
Ethanol (absolute)	AJAX-Sydney-Melbourne-Perth
CuSO ₄	Unilab AJAX-Sydney-Melbourne-Perth
2.4 Mercaptoethanol	Sigma St.Louis USA
MOPS (1)	Sigma St.Louis USA
MOPS (2)	Labiochem La Jolla USA

CHAPTER 3

INTRODUCTION

Determination of phytochrome pelletability and Pfr content in isolated subcellular fractions or excised plant tissues constitute a major portion of the data in this thesis. There are methodological problems associated with these determinations.

CHAPTER 3

may not be generally appreciated. During the course of my research, a number of problems were uncovered.

PROBLEMS OF METHODOLOGY AND

In this chapter I shall describe some of the problems encountered and the improvements developed for their rectification, in the determinations of total phytochrome, Pfr contents, and phytochrome pelletability.

THEIR RECTIFICATION

PHYTOCHROME MEASUREMENTS

The Spectrophotometric assay

The conventional spectrophotometric assay for phytochrome used in this study relies on the spectral properties of this molecule. As already pointed out in the general introduction, the absorption spectra of the two isomeric forms of phytochrome overlap considerably with maxima of absorbance near 680m and 730m for Pr and Pfr respectively. The two forms are therefore interconvertible most efficiently by light of these wavelengths. The conversion is however partial, and reaches a photoequilibrium

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INTRODUCTION

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PHYTOCHROME MEASUREMENTS

The Spectrophotometric assay

The conventional spectrophotometric assay for phytochrome used in this study relies on the spectral properties of this molecule. As already pointed out in the general introduction, the absorption spectra of the two isomeric forms of phytochrome overlap considerably with maxima of absorbance near 660nm and 730nm for Pr and Pfr respectively. The two forms are therefore interconvertible most efficiently by light of these wavelengths. The conversion is however partial, and reaches a photoequilibrium

at which the proportion of Pfr and Pr present is called the photostationary state for Pfr and Pr : $[Pfr]_{\infty}^{\lambda}$ and $[Pr]_{\infty}^{\lambda}$.

Dual wavelength spectrophotometers generally used to assay phytochrome are equipped with an irradiation system which produces high intensity red and far-red irradianations. These irradianations, called actinic irradianations, are capable of rapidly converting phytochrome in a sample so that, after a sequence of actinic irradianations, Pr or Pfr predominates according to the photostationary state established by the last actinic irradiation. A sample in which Pr predominates will have a higher 660nm and lower 730nm absorbancy (A) than a sample where Pfr predominates. It follows that a sample containing phytochrome will show photoreversible absorbancy changes at these wavelengths following a sequence of red and far-red actinic irradianations. The magnitude of these changes is directly related to the phytochrome content of the sample.

The dual wavelength spectrophotometers used to assay phytochrome and commonly referred to as 'Ratiospects' are designed to measure differences in absorbancy between either 660nm and 730nm, or between either wavelength and a reference wavelength at which absorbancy of phytochrome is negligible, or the same for Pr and Pfr; i.e. 800nm, the isosbestic point.

To minimise interference by the non-photoreversible absorbancy increase in the red region caused by transformation of protochlorophyllide to chlorophyllide following red

irradiation, the absorbancy difference measurements are usually made between 730nm and 800nm: $\Delta A = A_{730} - A_{800}$.

The relative amount of phytochrome and proportion of Pfr present in a sample are estimated by the difference between ΔA readings following red and far-red actinic irradiations, as will be described in the next section:

$$\Delta(\Delta A) = \Delta A_{fr} - \Delta A_r.$$

These absorbancy measurements are influenced by the scattering properties of a sample. Hence it is difficult to compare phytochrome contents of different materials *in vivo* or *in vitro*. In aqueous solutions, however, this difficulty can be minimised by the addition of a prescribed amount of CaCO_3 as a scattering agent, which also has the effect of increasing the optical path-length to several times the sample depth (Butler and Norris 1960).

Total Phytochrome and Pfr measurements

The determination of the proportion of phytochrome present in a sample as Pfr ($\text{Pfr}/\text{P}_{\text{total}}$) is entirely dependent on the photostationary state established by the actinic lights used in the Ratiospect, because all measurements are made on red and far-red irradiated samples. This will become apparent below.

Let us consider that a sample containing a mixture of Pr and Pfr gives an initial ΔA reading ΔA_1 (Fig. 3.1). After a far-red irradiation, which transforms almost all

FIG. 3.1

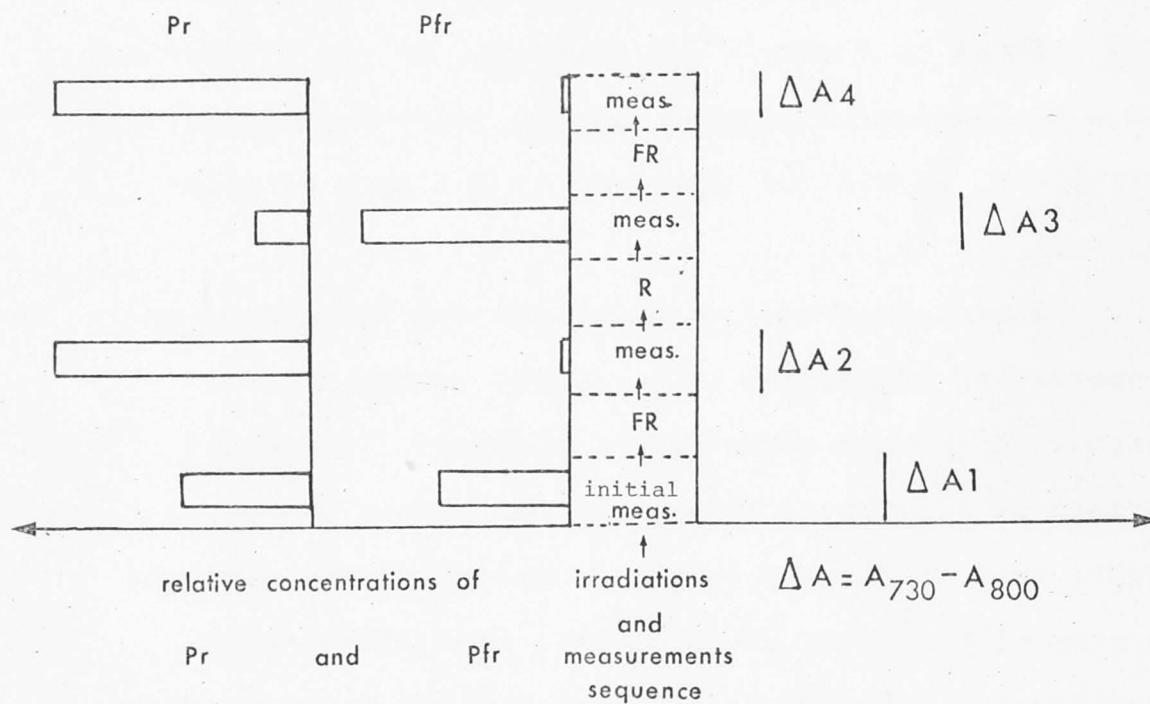
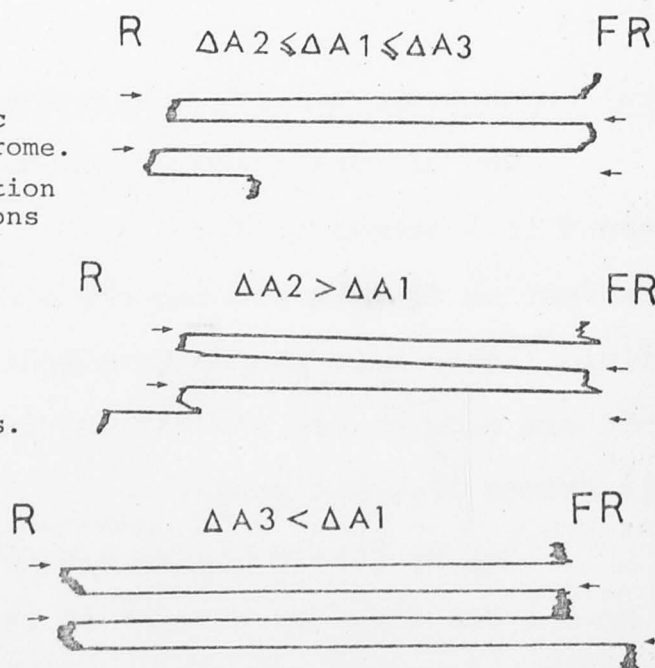


Fig. 3.1 Spectrophotometric assay for phytochrome.

Above: schematic representation of the relative concentrations of Pr and Pfr, with the corresponding absorbancy measurements in a sequence of actinic irradiations.

Right: chart recording showing 'normal' readings (top) and anomalous readings.



Pfr to Pr, the absorbancy at 730nm will decrease and the difference $\Delta A_2 = (A_{730} - A_{800})$ will be smaller. A subsequent irradiation with red light will drive the phytochrome in the sample to a new photoequilibrium mixture of Pr and Pfr, characteristic of the wavelength produced by the red actinic irradiation. The absorbancy at 730nm will increase and the resultant ΔA_3 will be larger than ΔA_2 and larger than or equal to ΔA_1 (see Fig. 3.1).

Thus the fraction of phytochrome initially present as Pfr : P_1 , is given by the general equation:

$$P_1 = \frac{[\Delta A_1 - \Delta A_2] \cdot [P_3 - P_2]}{[\Delta A_3 - \Delta A_2]} + P_2 \quad 3(1)$$

while

$$P_{\text{tot}} \propto \frac{\Delta A_3 - \Delta A_2}{P_3 - P_2} \quad 3(2)$$

where P_2 and P_3 are the photostationary states for Pfr established by the far-red and red actinic irradiations respectively and expressed in percent.

Because Pr does not absorb significantly in the far-red region, the far-red irradiation will transform essentially all phytochrome to the Pr state (i.e. $P_2 \sim 1-2\%$). Therefore we can assume $P_2 = 0\%$ in equations 3(1) and 3(2). On the other hand, because Pfr does absorb considerably in the red region the conversion of phytochrome to Pfr by red irradiation is partial and P_3 , the percentage of phytochrome present as Pfr after red actinic irradiation, must be derived

from photoconversion kinetics and absorption data of purified preparations of phytochrome.

The methodology and theoretical considerations involved in the determination of the photostationary state established by red light have been described in detail by Butler *et al.* (1964a) and Butler (1972). They derived photostationary states of 0.81 and 0.19 for Pfr and Pr respectively in red light ($[Pfr]_{\infty}^{665}$, $[Pr]_{\infty}^{665}$).

Calculations of Pfr content in intact tissues or aqueous solutions of phytochrome have since been based on these values (see Pratt 1975b for references).

Equations 3(1) and 3(2) can thus be simplified to:

$$P_1 = \%Pfr = \frac{\Delta A_1 - \Delta A_2}{\Delta A_3 - \Delta A_2} \cdot 80 \quad 3(3)$$

and

$$P_{tot} = \frac{\Delta A_3 - \Delta A_2}{80} \cdot k \quad 3(4)$$

where k is a constant of proportionality. The calculations of Pfr content in experiments described in this thesis are based on equations 3(3). To compensate for base line drifts, $\Delta(\Delta A)$ measurements are made following two cycles of red and far-red actinic irradiations, and one unit of phytochrome as defined here, corresponds to that amount of phytochrome giving a $\Delta(\Delta A)$ reading of 2.5×10^{-4} .

The values reported by Butler and associates, however, were derived from measurements made on a purified but degraded

(Pratt 1975a) phytochrome preparation. Pratt (1975a, b) has repeated the measurements on degraded and undegraded purified phytochrome from oats. He confirmed the values reported by Butler *et al.* (1964a) for degraded phytochrome, and calculated values of $[Pfr]_{\infty}^{665} = 0.75$ and $[Pr]_{\infty}^{665} = 0.25$ for undegraded phytochrome. He also pointed out that this value is likely to be affected by slight changes in the absorption properties of Pr and Pfr (Pratt 1975b), which could have occurred on its isolation and purification. Indeed, spectral shifts in the wavelength of maximal absorption have been reported for phytochrome *in vitro* and *in vivo*, following different light treatments (Kendrick and Roth-Bejerano 1978); furthermore, even within the intact tissues, the wavelength for absorption maximum of phytochrome following red irradiation can vary in accordance with a circadian rhythm (Horwitz and Epel 1978). Because of these uncertainties, I have chosen to retain the value of 0.81 for $[Pfr]_{\infty}^{665}$, for calculations of Pfr content.

Some technical problems associated with phytochrome measurements and their rectification

Provided that the red and far-red actinic beams in the Ratiospect establish photostationary states for Pfr of about 81% and 1% respectively, then samples containing between 1% and 81% Pfr should give ΔA measurements such that $\Delta A_1 > \Delta A_2$ and $\Delta A_3 > \Delta A_1$, when they are assayed for phytochrome content. I have however encountered cases where $\Delta A_2 > \Delta A_1$ and $\Delta A_3 < \Delta A_1$, as described below.

1. Cases where $\Delta A_2 > \Delta A_1$

Cases where ΔA_2 following FR actinic irradiation was greater than the initial ΔA_1 , were encountered when phytochrome was predominantly present as Pr in the sample analysed. For example, in intact tissues or in subcellular fractions derived from seedlings cultivated in darkness or irradiated with a wavelength known to establish a low photostationary state. For instance, a sample of FR irradiated material (presumably containing ~1% Pfr) would give ΔA values such that $(\Delta A_1 - \Delta A_2) / (\Delta A_3 - \Delta A_2) = -15/77$ units, instead of the expected 0/77 units. Hence the initial difference in absorbancy measured between 730nm and 800nm (ΔA_1) has increased following the first far-red actinic irradiation (ΔA_2). This anomaly can be best explained if the first FR actinic irradiation transforms some of the phytochrome, initially present as Pr, to the Pfr form: the FR light thus establishing a photostationary state significantly greater than 1%.

A spectral examination of the combination of the manufacturer's filter combination (filter A) originally used to provide the FR actinic beam was carried out. The transmission spectrum of this filter assembly is shown in Fig. 2.2 and reveals a non-negligible transmission of light below 700nm. Light of this wavelength would account for significant phototransformation of phytochrome to the Pfr form during the FR actinic irradiation. Hence the photo-

stationary state obtained would be significantly more than 1% Pfr, accounting for the anomaly. The cut-off filter in filter assembly A was therefore replaced by another which did not transmit below 700nm. The transmission spectrum of the new combination removed the leak below 700nm, and also removed the anomalous results of $\Delta A_2 > \Delta A_1$.

It should be pointed out that in a hypothetical situation where the phytochrome in a sample is all in the Pr form, the first FR actinic irradiation would increase the Pfr content of this sample from 0% to 1%. Therefore one would here expect measurements such that e.g.

$$(\Delta A_1 - \Delta A_2) / (\Delta A_3 - \Delta A_2) = -1/79 \text{ according to equation 3(1).}$$

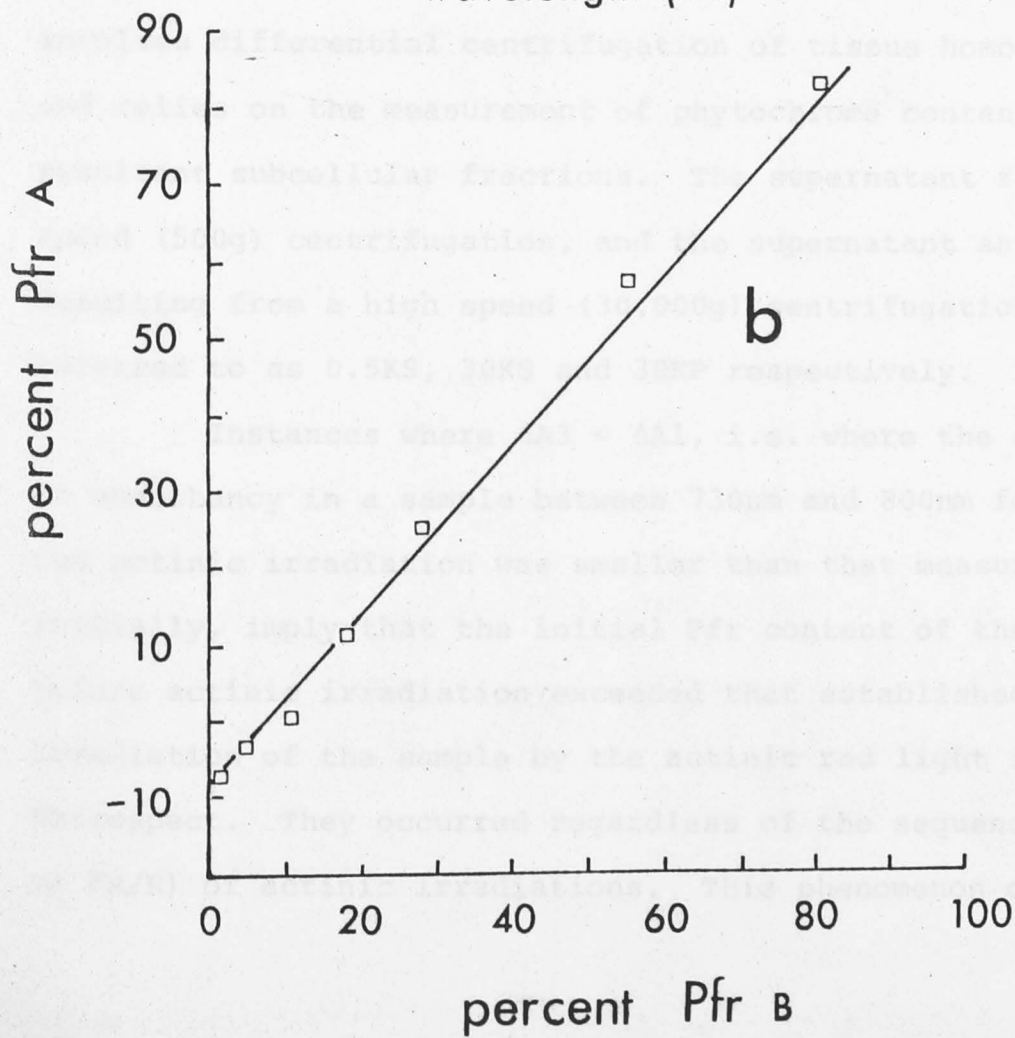
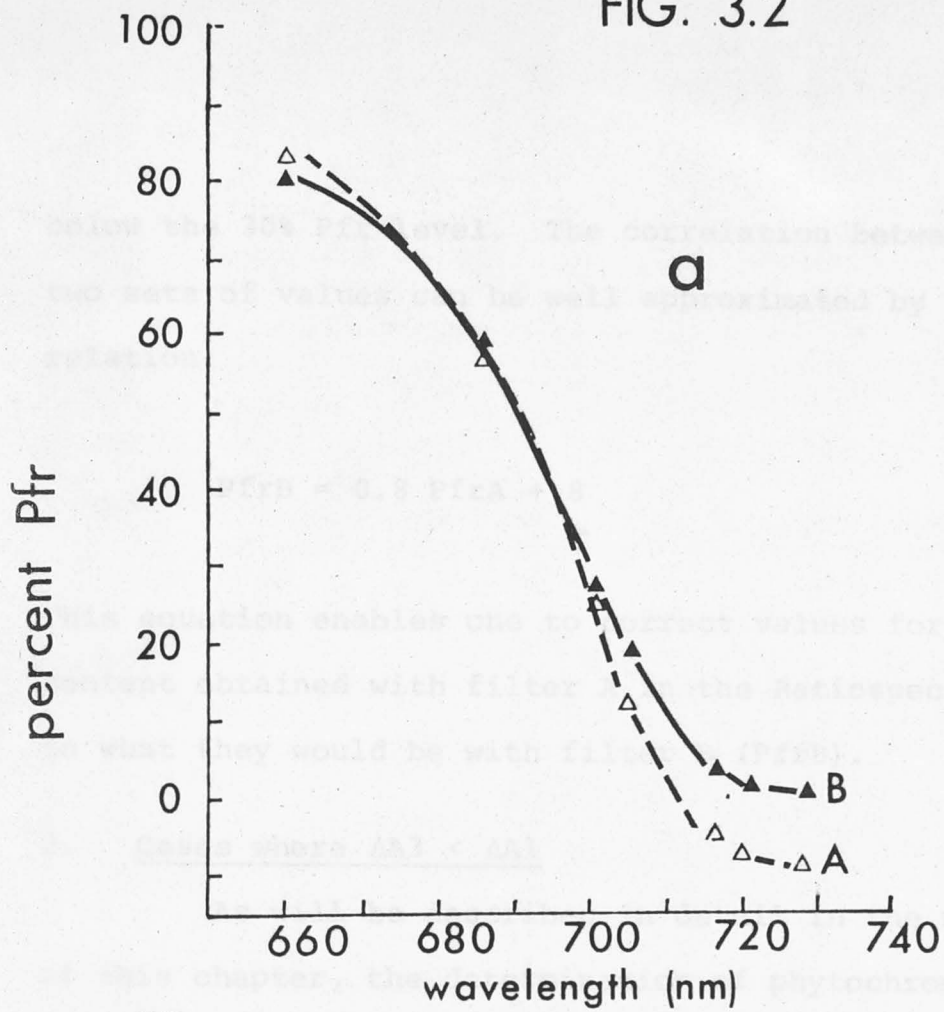
This small negative value would be well within the noise limit (typically 1-1.5 units) of the Ratiospect however, and would therefore not be easily or readily detected. The signal to noise ratio in the instrument limits the accuracy of Pfr measurements to 1-2%, for a sample containing 75-100 units of phytochrome.

Correlations between Pfr contents using filters A and B.

A comparison of Pfr contents derived from measurements obtained using filters A and B is shown in Fig. 3.2a. The correlation between these two sets of values is illustrated in Fig. 3.2b. Different Pfr levels were established by irradiating samples with light of different wavelengths. Figure 3.2a shows that above 30% Pfr, there is no significant difference between values obtained using filter assembly A or B, but the difference becomes significant

Fig. 3.2 a) comparison between Pfr contents measured using filter A or filter B to produce far-red actinic irradiations in the Ratiospect. Pfr was measured *in vivo* following saturating irradiations at the wavelengths shown.
b) correlations between Pfr measurements obtained using filter A (Pfr A) and filter B (Pfr B).

FIG. 3.2



below the 30% Pfr level. The correlation between these two sets of values can be well approximated by the linear relation:

$$\text{PfrB} = 0.8 \text{ PfrA} + 8 \quad 3(5)$$

This equation enables one to correct values for Pfr content obtained with filter A in the Ratiospect (PfrA) to what they would be with filter B (PfrB).

2. Cases where $\Delta A_3 < \Delta A_1$

As will be described in detail in the next section of this chapter, the determination of phytochrome pelletability involves differential centrifugation of tissue homogenates, and relies on the measurement of phytochrome content in the resultant subcellular fractions. The supernatant from a low speed (500g) centrifugation, and the supernatant and pellet resulting from a high speed (30,000g) centrifugation are referred to as 0.5KS, 30KS and 30KP respectively.

Instances where $\Delta A_3 < \Delta A_1$, i.e. where the difference in absorbancy in a sample between 730nm and 800nm following red actinic irradiation was smaller than that measured initially, imply that the initial Pfr content of the sample before actinic irradiation exceeded that established following irradiation of the sample by the actinic red light in the Ratiospect. They occurred regardless of the sequence (R/FR or FR/R) of actinic irradiations. This phenomenon occurred

Table 3.1 Pfr measurements in 30KP fractions, as a function of pH and Mg^{++} concentration of the buffer used for resuspension.

SRB at various pH			SRB + various $[\text{MgCl}_2]$		
pH	$\Delta\text{A1}-\Delta\text{A2}$	$\Delta\text{A3}-\Delta\text{A2}$	$[\text{Mg}^{++}]$ mM	$\Delta\text{A1}-\Delta\text{A2}$	$\Delta\text{A3}-\Delta\text{A2}$
6.95	64	53	0	87	71
7.1	61.5	53	1	91	80
7.3	57	47	5	98	98
7.5	62	51	10	100	100

when measurements of Pfr content were made on the resuspended 30KP obtained by fractionation of red irradiated coleoptiles, and also when the measurements were made on the same fraction after *in vitro* irradiation with red light. It seems also that this anomaly $\Delta A_3 < \Delta A_1$ is peculiar to the 30KP because it has not been observed with samples of 30KS or 0.5KS under the same conditions.

A number of physicochemical parameters were considered as possible causes for this anomaly. One of these, the nature of the buffer used to resuspend the 30KP pellet, was found to influence the ΔA measurements. When extraction buffer (SEBM) was used instead of the resuspension buffer (SRB), the anomaly, $\Delta A_3 < \Delta A_1$, was removed. Buffers used to homogenise tissues and resuspend 30KP pellets differ in pH, Mg^{++} and buffer ion concentrations. Mg^{++} concentrations and pH were tested: 30KP pellets were resuspended in extraction and resuspension buffers of different pH and/or Mg^{++} concentrations. The resuspended pellets were irradiated with red light (661nm) and assayed for their Pfr content. The results are shown in Table 3.1. It appears that it is the availability of Mg^{++} ions which determines the anomalous occurrence of $\Delta A_3 < \Delta A_1$ and therefore $(\Delta A_3 - \Delta A_2) < (\Delta A_1 - \Delta A_2)$, since pH did not affect this occurrence (Table 3.1).

The anomaly $\Delta A_3 < \Delta A_1$ was therefore removed by including Mg^{++} in the buffer used for resuspension of the 30KP. The physicochemical basis for its occurrence in samples

of red irradiated resuspended pellet, when Mg^{++} is excluded from the resuspension buffer, remains to be ascertained. This anomalous measurement means that the absorbancy difference between 730nm and 800nm ($A_{730} - A_{800}$) decreases following red actinic irradiation and that this decrease is a function of Mg^{++} concentration in the resuspension buffer.

It is unlikely that a Mg^{++} dependent absorbancy increase at 800nm can occur, since neither phytochrome nor other plant pigments absorb significantly in this region (see Chichester 1972, Goodwin 1976).

There are several possible causes for a decrease in absorbancy at 730nm, however.

i) The intense actinic red irradiation may partly bleach phytochrome, like chlorophyll (Jen and Mackiney 1970, Axelsson 1976), causing a loss of photoreversibility; this would imply that Mg^{++} protects phytochrome from such an effect. This possibility has not been investigated.

ii) The photostationary state for Pfr established by the actinic red irradiation in this sample, may be less than that obtained from the red light from the M3 monochromator used to irradiate samples prior to measurement.

If this is the case then the actinic red beam is either a) insufficiently intense for this sample, while adequate for others, e.g. 30KS, or b) the spectral quality of the red actinic beam is different from that obtained in the monochromator. Increasing the duration of the actinic red

irradiation causes A3 to be even less than A1; this effect is reversible which points to the second possibility and also rules out the possible interference by chlorophyll described by Jose *et al.* (1977).

The spectral transmission of the red interference filter used in the Ratiospect was checked and found to peak at 657nm, with narrow band, high transmission characteristics (Fig. 2.2). It differs from that used in the monochromator in the higher transmission characteristic only (Table 2.1). The filter therefore appears to be quite satisfactory, and indeed the anomalous measurements remained even after interchanging the filters between the monochromator and the Ratiospect.

The Ratiospect optical geometry has been inspected and it seems unlikely that divergent beams would be produced to affect the filter pass band. It is however impossible to analyse the spectrum of the red light produced at the sample position due to the combined geometry of the Ratiospect and measuring equipment. We can therefore not rule out the possibility that diverging beams or scattered light produce a small spectral shift of the actinic red beam in the Ratiospect with respect to that produced in the monochromator.

Assuming that a small shift may be present, the question arises whether the red actinic light can establish different photostationary states in samples of pellet resuspended in Mg^{++} -free or Mg^{++} -containing buffer. As pointed

out before (p. 35), this may be possible if Mg^{++} affects the absorbancy properties of phytochrome. I have therefore compared the spectral properties of pelleted phytochrome resuspended in the presence or absence of Mg^{++} , by means of differential spectroscopy between Pr and Pfr. The results are illustrated in Fig. 3.3. They show that the position of phytochrome absorbancy peak changes according to the availability of Mg^{++} in the resuspension buffer. Because the peak of Pfr is rather broad, shifts are not well defined. On the other hand, the absorption peak of Pr is distinct and the spectral shifts of the Pr peak position reported in Fig. 3.3 were consistently observed. Pr peak positions observed *in vivo* following different light treatments are also shown for comparison. Pr has an absorbancy peak situated at 667nm when pelleted phytochrome is resuspended in the absence of Mg^{++} . This peak position is shifted to 662nm when Mg^{++} is included in the resuspension buffer and is remarkably similar to that observed *in vivo* following a R/FR treatment, but not in non-irradiated tissue. As will be discussed later (p. 56) it is possible that the spectral shifts observed are related to the bound and free states of phytochrome.

Therefore, the possibility that the red actinic beam in the Ratiospect may establish different photostationary states for phytochrome in the 30KP, depending on the buffer used for resuspension, can not be excluded. This may provide an explanation for the anomalous measurements $\Delta A_3 < \Delta A_1$,

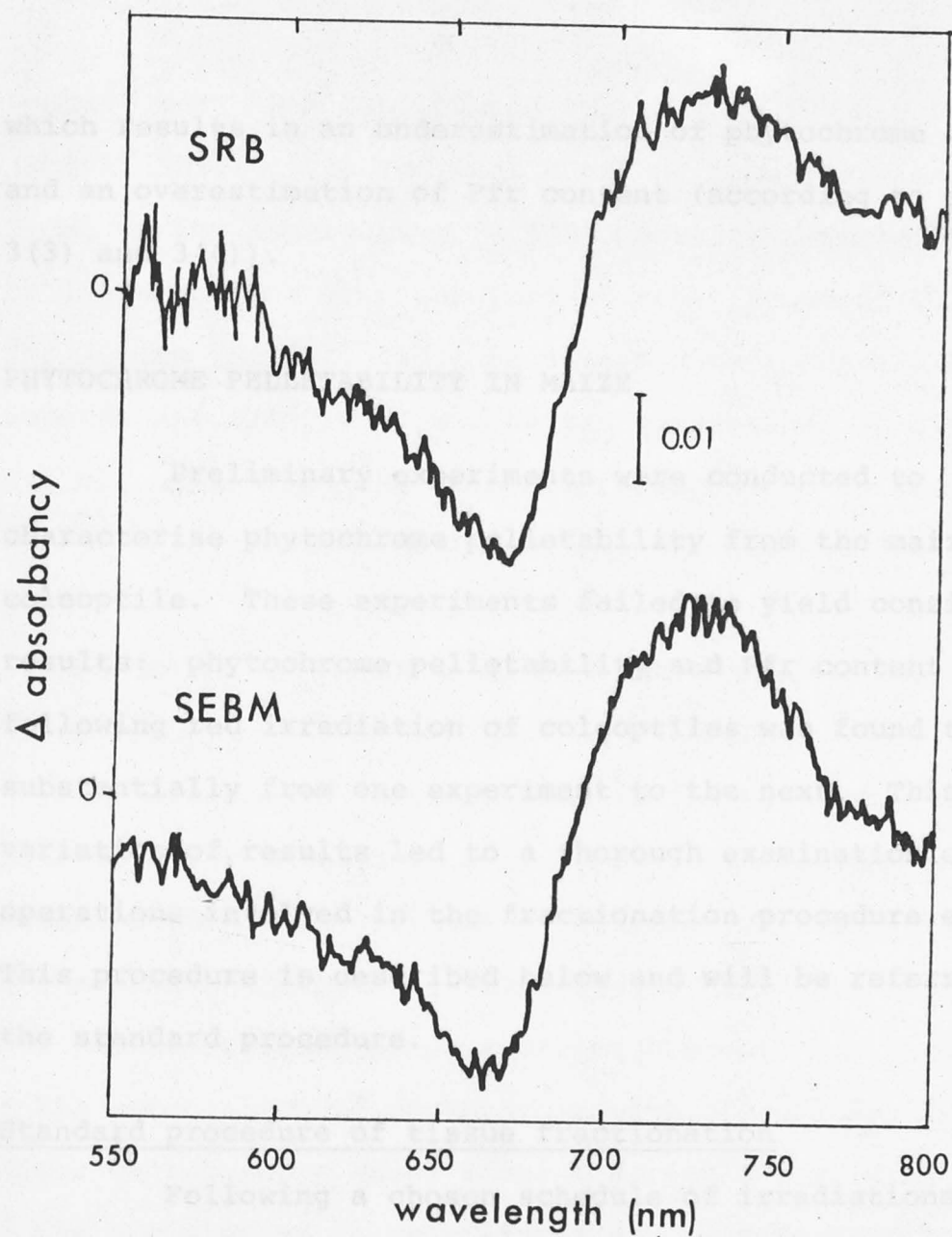
Fig. 3.3 Spectral characteristics of phytochrome
in vivo and *in vitro*.

In vitro: 10 g of freshly excised coleoptiles were irradiated with red light and homogenised in 32 ml SEBM. After fractionation by differential centrifugation, the resultant 30KP was resuspended in 1 ml SRB or SEBM, and further irradiated with FR light. Difference spectra were taken between these FR irradiated samples and samples further irradiated with white light.

In vivo: coleoptiles were chopped and packed into cuvettes 5 mm thick. Peak position for native Pr refers to the Pr peak position when the difference spectrum was established between coleoptiles irradiated with white light and unirradiated coleoptiles. Peak position for cycled Pr refers to the Pr peak position when the difference spectrum was taken between coleoptiles irradiated with white light and coleoptiles irradiated with white light followed by far-red light.

Difference spectra were taken at room temperature in a Cary 14 spectrophotometer equipped with an accessory for scattered transmission measurements (Cary 1462), and a manual base line correction. The data were analysed by a Tektronix computer programmed to subtract the base line from the difference spectrum every 0.4nm.

FIG. 3.3



Pr peak position (nm)			
in vitro		in vivo	
	30KP in		
SRB	667	native	655
SEBM	662	cycled	662

which results in an underestimation of phytochrome content and an overestimation of Pfr content (according to equations 3(3) and 3(4)).

PHYTOCHROME PELLETABILITY IN MAIZE

Preliminary experiments were conducted to characterise phytochrome pelletability from the maize coleoptile. These experiments failed to yield consistent results: phytochrome pelletability and Pfr content analysed following red irradiation of coleoptiles was found to vary substantially from one experiment to the next. This variation of results led to a thorough examination of the operations involved in the fractionation procedure employed. This procedure is described below and will be referred to as the standard procedure.

Standard procedure of tissue fractionation

Following a chosen schedule of irradiations and incubations, 1.25g of excised coleoptiles were immediately cooled by adding 4ml of extraction buffer (SEBM) at 0°C. The coleoptiles were chopped finely with a razor blade and homogenised by grinding in a pre-cooled pestle and mortar. The brei was filtered through one layer of nylon cloth. The final pH in all extracts was routinely 7.1. The filtered breis were then allowed to stand in ice until the last sample of the experiment was homogenised. The filtered breis of all

samples were then centrifuged together at 500 x g for 10 min (Sorval SS34). The sediment 0.5KP of each was discarded and 2.0ml of the supernatant (0.5KS), usually referred to as cell-free homogenate, was further centrifuged at 20,000g for 30 min (Sorvall SS34) to obtain a sediment (20KP) and supernatant (20KS). The 20KP was resuspended in either 0.5 ml, or 1 ml of the standard resuspension buffer SRB.

Phytochrome pelletability was expressed as:

$$B = 100 \times \Delta(\Delta A) \text{ in } 20KP / [\Delta(\Delta A) \text{ in } 20KP + \Delta(\Delta A) \text{ in } 20KS] \quad 3(6)$$

It should be clear that following this method the breis of early samples in a given experiment might have been incubated at 0°C for periods of varying duration, depending on the design of the whole experiment. This method assumes that parameters such as phytochrome content, Pfr concentration or the association of phytochrome with subcellular fractions remain unchanged in these samples during the waiting period of the breis prior to centrifugation. I have examined these parameters in cell-free homogenates as a function of the duration of incubation at 0°C.

Effect of *in vitro* incubation on Pfr content

Reversion of phytochrome from Pfr to Pr has been reported to take place in extracts from a number of higher plants (Butler *et al.* 1964b, Mumford 1966, Taylor 1968, Pike and Briggs 1972). To test whether this occurs in the cell-

FIG. 3.4

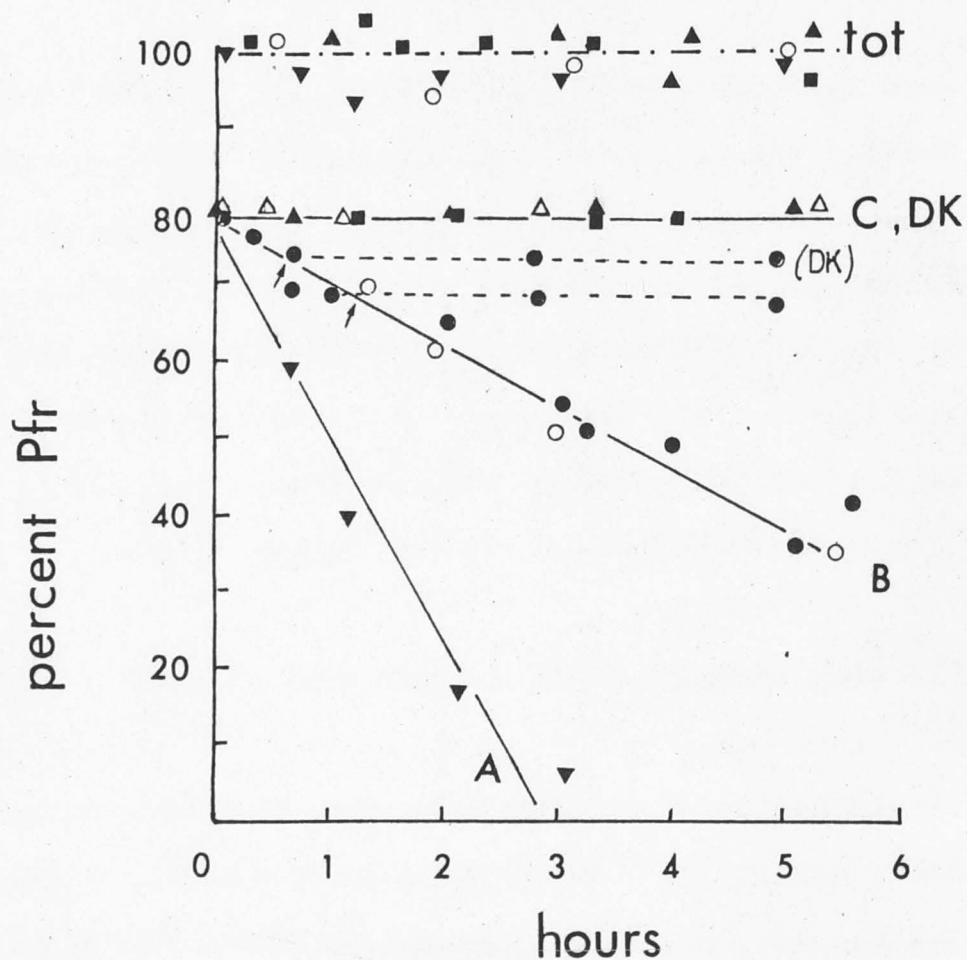


Fig. 3.4 Total phytochrome and Pfr content following 5 min red light, as a function of duration of incubation at 0°C under various safelights.

▼ : under SL Type A; ○, ● : under SL Type B;
 Δ, ▲ : under SL Type C; ■ in complete darkness;
 ----: after transfer to complete darkness at the time indicated by the arrow;
 --- : total phytochrome remaining in the sample at time t of incubation, expressed as a percentage of total phytochrome present at time zero.
 Open symbols: excised coleoptiles, *in vivo* measurements.
 Closed symbols: measurements in 0.5KS fractions.
 — : percentage Pfr.

free homogenates obtained from red irradiated maize coleoptiles, I have examined their total phytochrome and Pfr content as a function of time of incubation at 0°C under green safelights. The results are illustrated in Fig. 3.4. They show that, while phytochrome content remained essentially unchanged, Pfr content decreased following a first order kinetic with a half-life of 55 min, so that after 4 h no Pfr remained in the sample. The experiment was repeated in complete darkness to test whether this loss of Pfr was due to dark reversion of Pfr to Pr or was due to exposure of the sample to the safelights. The results in Fig. 3.4 demonstrate that no reversion of Pfr to Pr occurs in darkness in the extracts, and therefore that the safelights used were responsible for the conversion of Pfr to Pr and thus were not safe in this respect.

Conversion of Pfr to Pr by exposure to type A green safelights was due to emission of far-red light (see Fig. 2.1). Replacement by type B reduced the rate of conversion significantly. The complete removal of far-red emission which was achieved in type C green safelights prevented any conversion of Pfr to Pr. Incidentally, the conversion (if any) of Pfr to Pr by type B and C safelights was also investigated *in vivo* (Fig. 3.4). The results obtained *in vitro* were confirmed *in vivo* when red irradiated coleoptiles were incubated at 0°C under the 2 types of safelights.

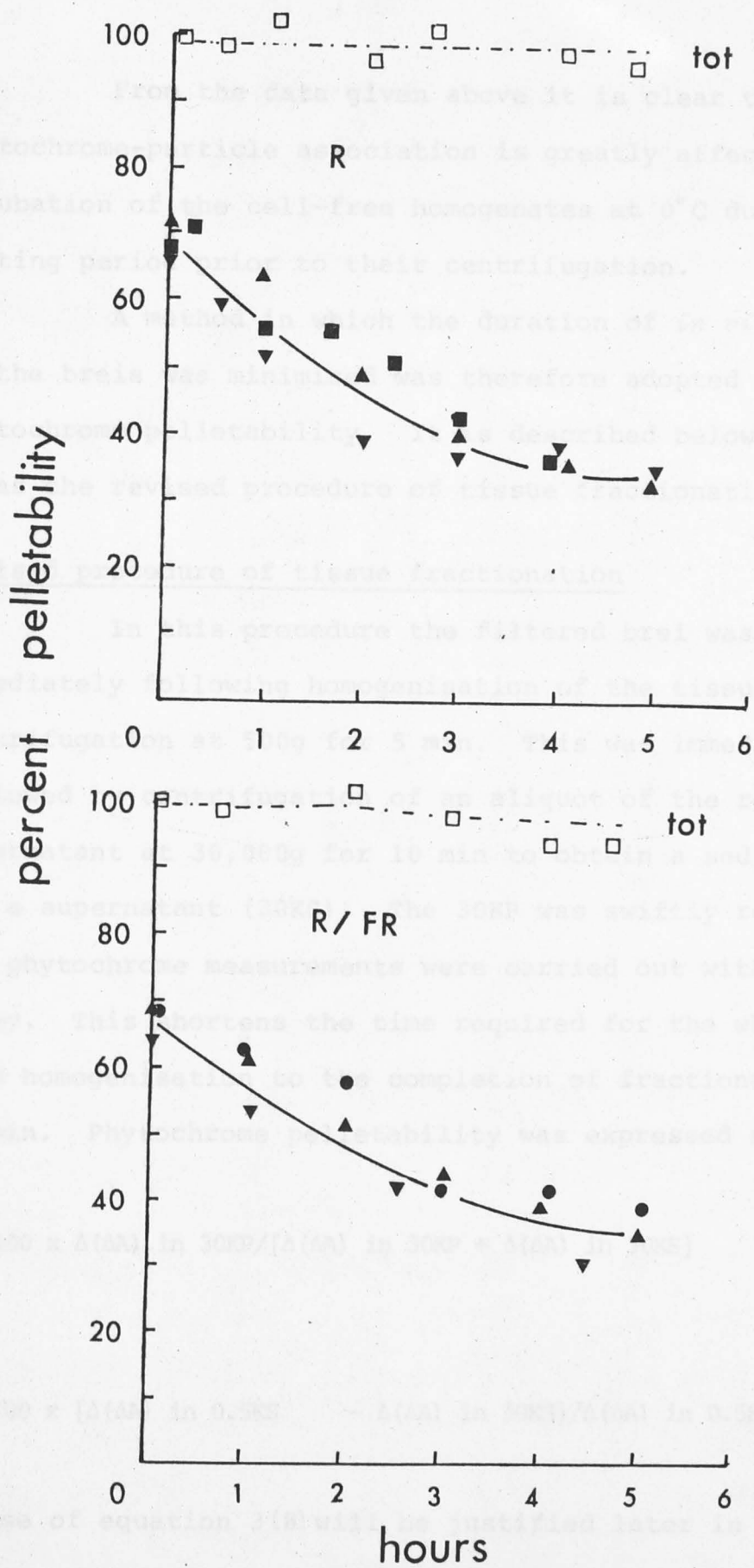
Stability of the phytochrome-particle association *in vitro*

Two parameters, phytochrome content and Pfr content, have been examined above. The third parameter investigated was the stability of the phytochrome-particle association *in vitro*. It is generally believed that it is the Pfr form of phytochrome which is capable of interacting with subcellular particles (see Chapter 1). Since the results of the experiments described above showed that Pfr can be converted to Pr by exposure to safelights, it was imperative to check whether or not the Pfr to Pr transformation might affect the stability of the association between phytochrome and the particulate fraction, during incubation at 0°C.

The following experiment was therefore conducted. Cell-free homogenates of R and R/FR irradiated coleoptiles were incubated for various periods of time at 0°C, in complete darkness or under the 3 types of safelights A, B, C. After incubation, they were fractionated as described above and phytochrome pelletability was determined. The results illustrated in Fig. 3.5 demonstrate that phytochrome is progressively released from the particulate fraction *in vitro*. The data further show that the release is independent of the Pfr to Pr transformation. It occurs at the same rate irrespective of the type, A, B or C, of safelight under which the breis were incubated, or whether phytochrome was already present as Pr at the beginning of incubation, i.e. after R/FR light treatment *in vivo*.

Fig. 3.5 Phytochrome pelletability as a function of duration of incubation at 0°C of crude homogenates from Red (top) or R/FR (bottom) irradiated coleoptiles.
■ incubation in complete darkness,
▼ under SL type A, ▲ under SL type C.
□---□ total phytochrome remaining in the sample at time t of incubation, expressed as a percentage of total phytochrome present at time zero.

FIG. 3.5



From the data given above it is clear that the phytochrome-particle association is greatly affected by the incubation of the cell-free homogenates at 0°C during the waiting period prior to their centrifugation.

A method in which the duration of *in vitro* incubation of the breis was minimised was therefore adopted for assaying phytochrome pelletability. It is described below and referred to as the revised procedure of tissue fractionation.

Revised procedure of tissue fractionation

In this procedure the filtered brei was fractionated immediately following homogenisation of the tissue by centrifugation at 500g for 5 min. This was immediately followed by centrifugation of an aliquot of the resultant supernatant at 30,000g for 10 min to obtain a sediment (30KP) and a supernatant (30KS). The 30KP was swiftly resuspended and phytochrome measurements were carried out with minimal delay. This shortens the time required for the whole procedure, from homogenisation to the completion of fractionation, to about 30 min. Phytochrome pelletability was expressed as:

$$B = 100 \times \Delta(\Delta A) \text{ in } 30KP / [\Delta(\Delta A) \text{ in } 30KP + \Delta(\Delta A) \text{ in } 30KS] \quad 3(7)$$

or

$$B = 100 \times [\Delta(\Delta A) \text{ in } 0.5KS - \Delta(\Delta A) \text{ in } 30KS] / \Delta(\Delta A) \text{ in } 0.5KS \quad 3(8)$$

The use of equation 3(8) will be justified later in this chapter.

FIG. 3.6

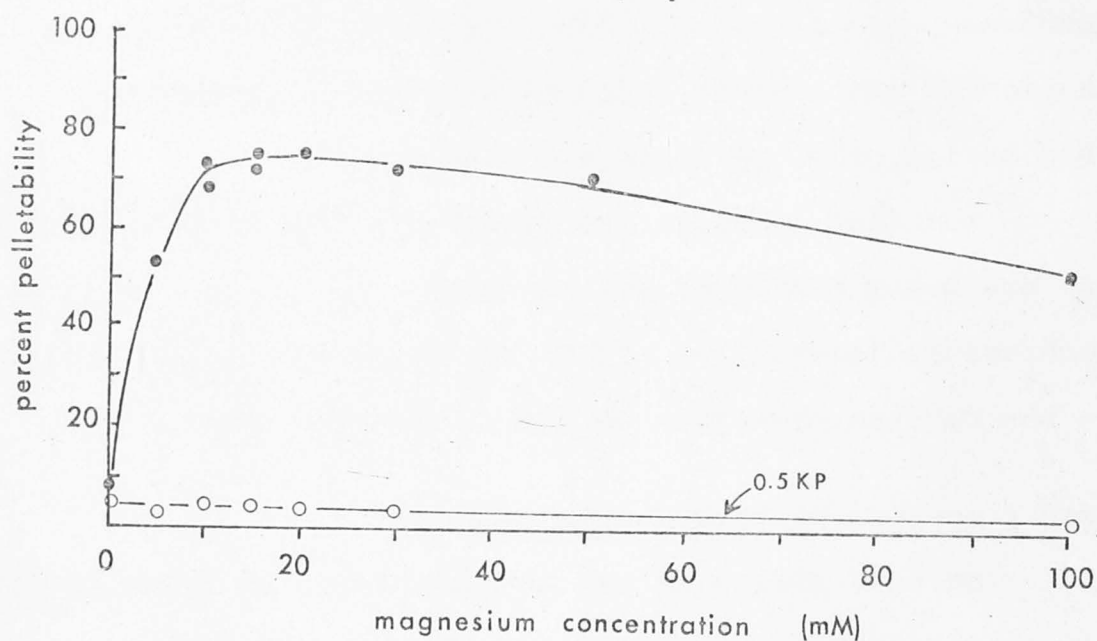


Fig. 3.6 Phytochrome pelletability following 5 min red *in vivo* irradiation, as a function of the Mg^{++} concentration of the homogenisation buffer.
 ●—● Pelletability calculated using equation 3(8).
 ○—○ Percentage of phytochrome in the 0.5KP.

This fractionation procedure was adopted in all experiments described in the following chapters.

Tests were made to determine whether the concentration of Mg^{++} (10mM) used in the extraction buffer is adequate to ensure maximal pelletability. Phytochrome pelletability in maize was studied as a function of the Mg^{++} concentration in the extraction medium, following 5 min R irradiation. Concurrently the first pellet (0.5KP) was kept for analysis of its phytochrome content. Results are shown in Fig. 3.6. Phytochrome pelletability in response to R irradiation increases as the Mg^{++} concentration of the extraction buffer increases up to about 10mM. At this concentration saturation of pelletability occurs. At very high ion concentrations (e.g. 100mM) pelletability decreases. The Mg^{++} dependency of phytochrome pelletability described here is in accordance with that found in other plant materials by Grombein *et al.* (1975b) and Pratt and Marmé (1976). However, unlike the situation in oats (Grombein *et al.* 1975b) and the claims of Pratt (1978) for maize, the phytochrome content of the low speed pellet (0.5KP) was negligible over the whole range of Mg^{++} concentrations examined, being less than 5% of the total phytochrome in the extract, and even lower under 20mM Mg^{++} .

It appears therefore that for studies of phytochrome pelletability in maize, 10mM as used in this procedure is an adequate concentration for the Mg^{++} ion in the extraction buffer.

FIG. 3.7

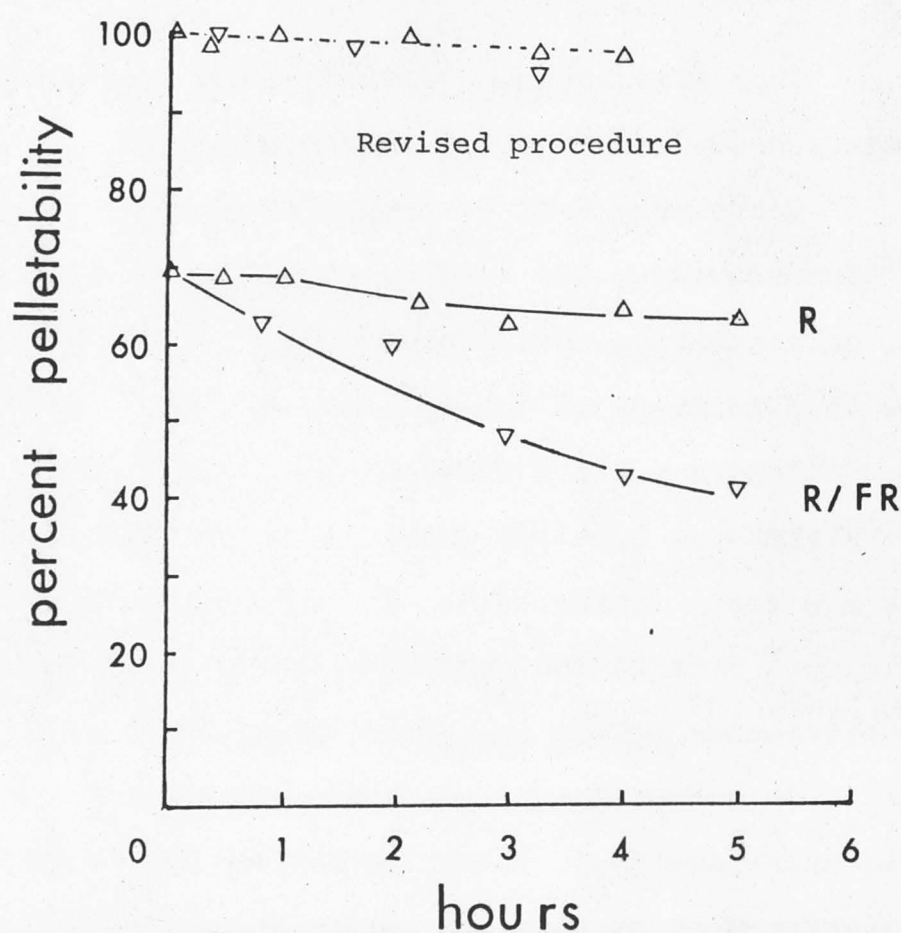


Fig. 3.7 Phytochrome pelletability as a function of duration of incubation of coleoptiles at 0°C following R (Δ) or R/FR (▽) irradiations. Phytochrome pelletability was determined using the revised procedure of tissue fractionation and equation 3(8).
 - - - - : total phytochrome expressed as a percentage of phytochrome content at time zero.

It should be stressed again that there is only one basic difference between the two procedures of tissue fractionation described here. In the standard procedure, cell-free homogenates may be subjected to an incubation of variable duration prior to fractionation whereas this is systematically avoided in the revised procedure.

In the following I should like to demonstrate how the instability of the phytochrome-particle association *in vitro* can affect the outcome of an experiment by comparing results of two experiments that were carried out using both the standard and the revised fractionation procedures.

1. Time course of subcellular distribution of phytochrome pelletability during *in vivo* incubation at 0°C

In this first experiment, pelletability of phytochrome was analysed in coleoptiles as a function of the duration of incubation at 0°C following a R or R/FR irradiation. Excised coleoptiles were irradiated with 5 min of R light or 5 min R followed by 5 min FR light. Following irradiation, they were incubated at 0°C. At scheduled times during incubation, samples were removed and processed for the determination of phytochrome pelletability. Results obtained using the revised or the standard procedure of tissue fractionation are shown in Figs. 3.7 and 3.8 respectively. These figures demonstrate that the two procedures yielded totally different results.

Fig. 3.7 shows that phytochrome becomes dissociated from the particulate fraction in R/FR treated material at a

FIG. 3.8

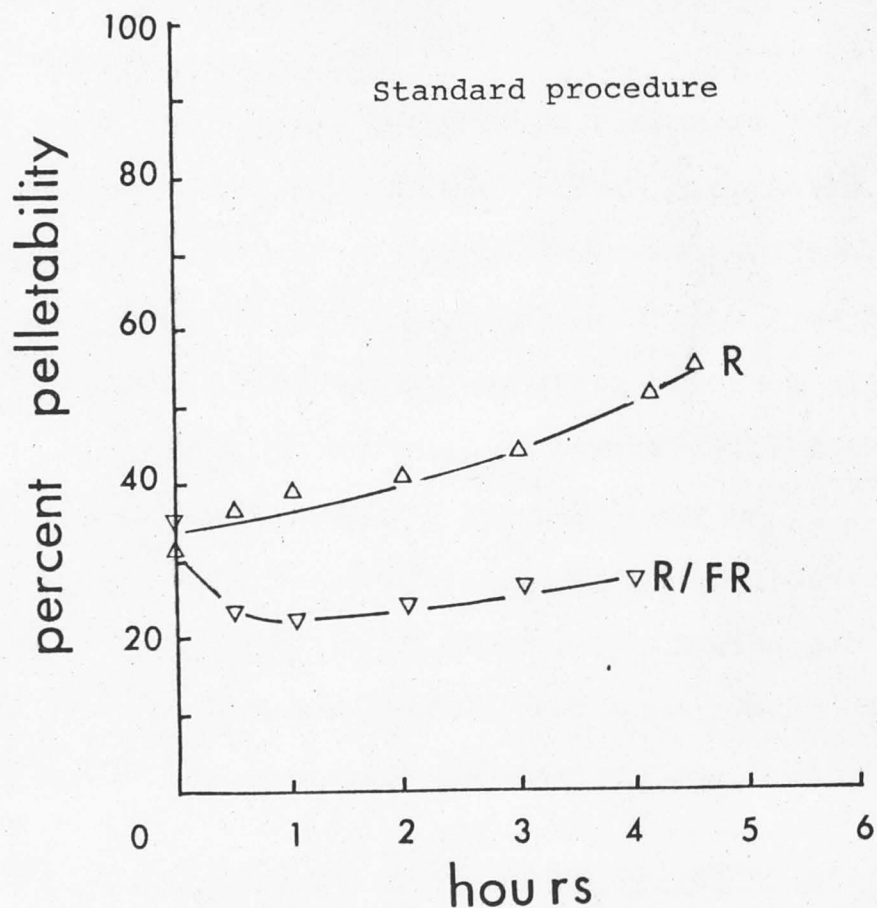


Fig. 3.8 Phytochrome pelletability as a function of duration of incubation of coleoptiles at 0°C following R(Δ) or R/FR(∇) irradiations.

Phytochrome pelletability was determined using the standard procedure of tissue fractionation and equation 3(6).

rate similar to that observed *in vitro*. In R irradiated material however, only a slow release is observed (so that after 6 h incubation at 0°C phytochrome pelletability is not much different from that at zero time of incubation).

When the same experiment was conducted using the standard fractionation procedure (Fig. 3.8), red irradiation resulted in an apparently continuous increase of phytochrome pelletability upon incubation of the irradiated coleoptiles at 0°C while R/FR irradiation resulted in little change: an apparent small decrease was followed by a slow increase.

As it will become apparent below, the data of Fig. 3.8 can be considered as a combination of the data of Fig. 3.7 and those of Fig. 3.5. Indeed, from the data of Figs. 3.7 and 3.5 one can calculate the results one would expect to obtain from R/FR irradiated coleoptiles during 4 h *in vivo* incubation at 0°C in the dark using the standard fractionation procedure (Fig. 3.8). These calculated values are presented in Table 3.2 and are very similar to the data of Fig. 3.8. The use of the standard fractionation procedure in this experiment implies that samples which could be labelled T0, T1, T2, T3 and T4 to represent *in vivo* incubation at 0°C for 0, 1, 2, 3 and 4 hours have in effect also been subjected to (at least) 5, 4, 3, 2 and 1 hours of incubation as breis at 0°C respectively. During this time phytochrome content would progressively decrease in the particulate fraction (see Fig. 3.5). For example, sample T0 has suffered loss of phytochrome from the particulate fraction during 4 h standing

Table 3.2 Comparison of the measured and calculated distribution of phytochrome during *in vivo* incubation at 0°C following R/FR irradiation, and using the standard fractionation procedure.

Duration of <i>in vivo</i> incubation	Corresponding duration of incubation of the brei at 0°C before phyto- chrome measurement	Residual pelletability $100\% \times B_t/B_0$ as calculated from data of Fig. 3.5	Taking $B = 60\%$, predicted B_t in %	Measured B_t from Fig. 3.8
0	5 h	52.5	31.3	32
30 min	4 h 30 min	45	27	23
1 hour	4 h	41	24.6	22.5
2 h	3 h	44.5	26.8	24
3 h	2 h	36.6	22	25
4 h	1 h	38.8	23	25.5

Abbreviation: B = percentage phytochrome pelletability;
 B_t , B_0 = pelletability time t and zero time
 respectively.

as a brei waiting for sample T4 to be harvested and homogenised, before centrifugation finally took place. On the other hand, the last sample of the experiment T4, which was incubated *in vivo* for 4 h only suffered 1 h *in vitro* incubation (homogenisation and fractionation taking approximately 1 h). It follows therefore that all samples were incubated for the same total time summing the *in vivo* and *in vitro* incubations.

In the case of R/FR irradiated coleoptiles, since phytochrome is released at similar rates *in vivo* (i.e. before homogenisation, Fig. 3.7) and *in vitro* (i.e. after homogenisation, Fig. 3.5), it is to be expected that all samples would show similar final values for phytochrome pelletability by the end of the experiment.

Another example illustrating the consequences of *in vitro* incubation prior to centrifugation is given by the experiment described below.

2. Characterisation of phytochrome pelletability

Phytochrome pelletability following R, FR or R/FR irradiation was investigated using either the revised or the standard procedure of tissue fractionation. In the latter case irradiation treatments during the course of the experiment were carried out in the following order: R/FR/R, R, FR, R/FR with 40 min interval introduced in the middle of the experiment. The results obtained are reported in Table 3.3. The two procedures again yielded two types of data: using the revised procedure, R, R/FR and R/FR/R irradiations were found to yield

Table 3.3

Irradiation treatment	Percentage pelletable phytochrome		
	Results obtained with Revised Procedure	Standard Procedure	Results of Quail <u>et</u> <u>al.</u> 1973
3 min R/5 min FR/ 3 min R	57.4	34.4	15.6
3 min R	56	35.2	15.9
5 min FR	7.4	7.4	5.7
3 min R/5 min FR	56.9	50.7	26.8

approximately the same pelletability levels, while using the standard procedure, R and R/FR/R irradiations apparently yielded lower pelletability levels as compared to R/FR irradiations. When the results obtained above are compared to those obtained by Quail *et al.* (1973a) in maize, it is apparent that the pattern of results obtained with the standard method is similar to that obtained by these authors.

CONCLUSIONS

In this chapter a number of technical problems encountered and ways of overcoming them have been described.

I shall briefly summarise these problems and show to what extent they affected the results of experiments carried out before their rectification. Finally, I shall discuss some preliminary results that were obtained in the process of rectification.

Faults in methodology as described in this chapter can be attributed to:

- a) the far-red component of the light transmitted by types A, B green safelights affecting the original Pfr content of a sample of phytochrome upon prolonged exposure.
- b) a red component in the actinic far-red source of the Ratiospect, affecting determinations of Pfr content, especially at low Pfr levels.
- c) the *in vitro* instability of the phytochrome-particle association, affecting the determination of phytochrome

pelletability.

- d) the dependency of an accurate assay of Pfr and total phytochrome contents in the 30,000 g pellet, on magnesium ions in the buffer used for resuspension.

Most of the problems discovered were rectified during the course of my study. Hence, some of the experiments were conducted under suboptimal and some under both optimal and suboptimal conditions. Data obtained prior to rectification of a particular problem had to be carefully revised and corrected for the few cases where time did not allow the appropriate experiment to be repeated under optimal conditions. All data presented in Tables and Figures in the following chapters include a statement of the experimental conditions and of the corrections applied, if any.

Reliable measurements of the Pfr contents of 30,000 g pellets were particularly difficult and could be not achieved until the problems summarised above were properly rectified. Pfr measurements made *in vivo* or in other subcellular fractions were less greatly affected however, since:

- a) as long as the safelights were not equipped with a specific far-red absorbing filter, the effects of the transmitted far-red light could be controlled and minimised in these samples by minimising their exposure to safelights. Ultimately, all handling of the samples could be carried out in complete

darkness; it is very difficult to resuspend 30KP fractions in these conditions, however. It should be pointed out here that type A safelights were removed very early and were not used in any experiments other than those already described (p. 45).

- b) problems associated with Pfr measurement caused by a red leak of the actinic far-red beam of the Ratiospect were simply and reliably overcome using equation 3(5).

The determination of phytochrome pelletability was the least affected because:

- a) the standard method of tissue fractionation was not used for experiments other than a few preliminary ones and those described only in this chapter.
- b) exposure to type B safelights does not affect phytochrome pelletability.
- c) determination of phytochrome pelletability does not necessarily rely on the measure of phytochrome content in the 30KP fraction. Indeed this value can be calculated from measurements of phytochrome content made on the 0.5KS and 30KS fractions and therefore percentage phytochrome pelletability can be calculated by equation 3(8).

Therefore, although the anomaly $\Delta A_3 < \Delta A_1$ was not resolved for a long time, this alternative procedure of calculation was found to be quite adequate: i.e. it yielded

the same results as when percentage pelletability was calculated from measurements made on 30,000 g pellets resuspended in SEBM using equation 3(7).

In the process of revising the method used for determinations of phytochrome pelletability, information regarding the characteristics of the phytochrome-particle association was obtained. Although the phytochrome-particle association is unstable *in vitro*, results shown in this chapter indicate that the situation is quite different *in vivo*. It appears that the stability of this association *in vivo* is different in R or R/FR irradiated tissues when they are incubated at 0°C in darkness. For instance, phytochrome pelletability in R/FR irradiated tissue decreases upon incubation at a rate similar to that occurring *in vitro*: this is not the case in R irradiated tissues, where the small decrease is very slow.

The dark *in vitro* release of phytochrome from a particulate fraction suggested that this association becomes unstable once the cellular organisation is disrupted. This phenomenon would argue against the possibility of an Mg^{++} -dependent preferential precipitation of Pfr during homogenisation as suggested by Quail (1975c). It supports rather the hypothesis that association of phytochrome with the potential particulate fraction is a light-induced intracellular reaction (Yu 1975a).

Another argument in favour of this hypothesis may come from a comparison of the spectral properties of Pr *in vivo* and *in vitro* (Fig. 3.3).

It was observed that the spectral characteristics of particulate Pr (30KP in SEBM) are similar to those of "potential" pelletable Pr (Pr *in vivo* following R/FR irradiation of the coleoptiles), while they are different from those of "soluble" Pr both *in vivo* (native Pr in dark tissue) and *in vitro* (pelleted Pr released from the particulate fraction by resuspension in Mg^{++} -free buffer (Marmé 1974).

The similarity above may be indicative of a similarity in the molecular state and/or molecular environment of "potential pelletable Pr" *in vivo*, and that of particulate Pr (*in vitro*) obtained after homogenisation, fractionation and resuspension of the resulting pellet in Mg^{++} -containing buffer. These observations are therefore suggestive of a stabilisation by Mg^{++} of a red (or R/FR) induced intracellular association of phytochrome with the particulate partner, otherwise labile during homogenisation of the tissue or resuspension of particulate fraction in the absence of Mg^{++} .

Also, the data presented by Quail (1978a) showing that rapid post-homogenisation addition of Mg^{++} to Mg^{++} -free extracts sustains enhanced pelletability of phytochrome but with rapidly declining effectiveness over 1-2 min after extraction, are consistent with this hypothesis.

On the other hand, Quail (1978a, b) found differences between Pr and Pfr regarding the *in vitro* stability of the phytochrome-particle association in oats, when analysed as a function of Mg^{++} concentration. In this chapter, no

differences between Pr and Pfr were found *in vitro* as both forms were released from the particulate fraction at the same rate. These contradictory observations may be explained if it is assumed that either i) phytochrome from *Zea* behaves differently than that from *Avena*, at least *in vitro*, and this is likely to be the case (see above p. 48 and below p. 58) and/or ii) that the time-dependent release of phytochrome from the pelletable condition *in vitro*, and in the presence of a constant concentration of Mg^{++} , is a reaction unrelated to the Mg^{++} -dependent stability of the phytochrome-particle association described by Quail (1978a, b).

In view of the results obtained for the *in vitro* and *in vivo* stability of the phytochrome and particle association, attempts were made to rationalise data reported by other workers in the field.

Manabe and Furuya (1975) reported that during the dark incubation of pea shoot segments following a brief red irradiation, the association of phytochrome with particulate fractions was significantly enhanced (3-4 times), whilst phytochrome destruction was inhibited in the presence of EDTA or dithiothreitol.

Using the revised procedure for tissue fractionation I was unable to observe any substantial increase in phytochrome pelletability during the dark incubation at 0°C of red irradiated coleoptiles, a treatment which also prevented phytochrome destruction (see Fig. 3.7). Using the standard

procedure however, an apparently continuous increase of phytochrome pelletability was obtained (Fig. 3.8) following red irradiation. Recognising that dark incubation of the brei at 0°C causes the release of particulate phytochrome, it follows that the increase of pelletability described is in fact not real, but only an artefact of the technique used. Due to uncertainty regarding experimental details described in their report, and to the different plant material used, it is not possible to evaluate the physiological significance of the increase of pelletability obtained by Manabe and Furuya (1975). It is possible that the *in vitro* decrease of pelletability observed with maize may not be a general phenomenon. This is supported by the fact that Pratt and Marmé (1976) and Kendrick and Roth-Bejerano (1978), working on oat shoots and barley primary leaves respectively, reported that the *in vitro* incubation of pelletable phytochrome as Pr (i.e. following far-red irradiation *in vitro*) does not yield a measurable decrease of pelletability over a two hour period.

It is tempting to assume, however, that the data reported by Quail et al. (1973a) may suffer from such an artefact caused by the *in vitro* instability of the phytochrome-particle association reported here for maize. Indeed, working on the same plant material as they used, a pattern of results similar to that reported by these authors could be obtained, under conditions where a waiting period for the breis prior to centrifugation was voluntarily included (Table 3.3). The

details of the experiment of Quail *et al.* are not sufficient to conclude that an analogous waiting period was inadvertently included in their experiment. It remains however that using the revised method, R/FR irradiation of maize coleoptiles has consistently failed to yield the significantly higher pelletability than that following R or R/FR/R irradiation reported by Quail *et al.* (1973a).

CHAPTER 4

ACTION SPECTRUM FOR THE INDUCTION OF PHYTOCHROME PELLETABILITY

CHAPTER 4

INTRODUCTION

When plant tissues are irradiated with red light, phytochrome becomes pelletable upon homogenization and fractionation of the tissue (see Chapter 1 and results of Chapter 3).

CHAPTER 4

ACTION SPECTRUM FOR THE
INDUCTION OF PHYTOCHROME
PELLETABILITY

It is often but not experimentally demonstrated, that because of its presence in the particulate fraction following homogenization, phytochrome is indeed the photoreceptor for the induction with this particulate fraction (Quail and Schäfer 1974, Schäfer 1975b). At the time of starting this work, when the usual criterion for the involvement of phytochrome in a response was applied, i.e. a reversal of the red induced effect by far-red light (Wong 1972), immediate reversal of phytochrome pelletability as induced by 3-5 min red light was not usually obtained (Quail et al. 1973a, Quail and Schäfer 1974, Table 2.1). Although not derived from a critically designed test (see below), this observation could be said to raise doubts as to whether or not phytochrome is actually the primary photoreceptor for its own induction of pelletability. It is conceivable that phytochrome may not be the primary photoreceptor, but may be involved subsequently due to light absorption by another pigment. In contrast to other red induced photoreponses in plants, no action spectra for the

CHAPTER 4

INTRODUCTION

When plant tissues are irradiated with red light, phytochrome becomes pelletable upon homogenisation and fractionation of the tissue (see Chapter 1 and results of Chapter 3).

It is often assumed, but not experimentally demonstrated, that because of its presence in the particulate fraction following red light irradiation, phytochrome is indeed the photoreceptor for its own association with this particulate fraction (Quail and Schäfer 1974, Schäfer 1975b). At the time of starting this work, when the usual criterion for the involvement of phytochrome in a response was applied: i.e. a reversal of the red induced effect by far-red light (Mohr 1972), immediate reversal of phytochrome pelletability as induced by 3-5 min red light was not usually obtained (Quail *et al.* 1973a, Quail and Schäfer 1974, Table 2.1). Although not derived from a critically designed test (see below), this observation could be said to raise doubts as to whether or not phytochrome is actually the primary photoreceptor for its own induction of pelletability. It is conceivable that phytochrome may not be the primary photoreceptor, but may be involved subsequently due to light absorption by another pigment. In contrast to other red induced photoresponses in plants, no action spectra for the

light-induction of phytochrome pelletability have been reported. I have therefore determined the action spectrum for phytochrome pelletability in etiolated maize coleoptiles, from 400nm to 730nm. In addition, the effect of prolonged irradiation on phytochrome pelletability was investigated as a function of wavelength to see whether or not, as in some classical phytochrome-mediated responses (e.g. anthocyanin synthesis and inhibition of hypocotyl lengthening in lettuce, see Mohr 1964), sustained irradiation with far-red light can mimic the action of short irradiation with red light.

EXPERIMENTAL

Irradiation procedure

Monochromatic light was obtained using the monochromator and interference filters described in Chapter 2. Unless otherwise stated, irradiances expressed in Wm^{-2} at the wavelengths used were as follows: 425nm: 0.30; 447nm: 0.65; 473nm: 2.1; 509nm: 2.9; 532nm: 2.0; 552nm: 1.15; 578nm: 1.17; 621nm: 2.05; 661nm: 0.25; 675nm: 0.25; 685nm: 1.40; 694nm: 1.3; 706nm: 0.8; 717nm: 0.8; 727.5nm: 0.75; 730nm: 0.7. For sustained irradiation (4 h), irradiances at 661nm and 675nm were 1.65 and 1.40 Wm^{-2} respectively, and 4 ml of buffer (SEBM without mercaptoethanol) was added to prevent dehydration of the coleoptiles. Irradiations of excised coleoptiles were carried out at room temperature ($\sim 20^\circ\text{C}$).

Other experimental conditions

The safelights used in experiments in which phytochrome pelletability only was analysed were type B. Experiments involving Pfr measurements were carried out either under type C safelights, or in complete darkness, except for the actinic irradiation treatments. Pfr measurements were made either with filter B in place in the Ratiospect, or filter A and corrected using equation 3 (5), as indicated in the relevant Figures. Pelletability values were calculated using equation 3 (8). From this it follows that the Figures presented in this chapter are slightly different from those already published (Fuad and Yu 1977).

RESULTS

Dose response curves

In constructing an action spectrum of photo-induced phytochrome pelletability in the visible range, dose response curves were determined for a selected number of wavelengths between 420nm and 730nm. The different light doses for all wavelengths selected were obtained by varying the duration of irradiation at a chosen light intensity, while other experimental conditions remained essentially the same. Figure 4.1a shows a typical dose response curve in which percentage pelletability is plotted against duration of irradiation. The shape of the curve appears to be exponential

Fig. 4.1 Dose response curves for the induction of phytochrome pelletability. Irradiations were performed on excised coleoptiles. Pelletability was calculated using equation 3(8).⁻². Irradiance at 661nm was 0.25 Wm⁻².

FIG. 4.1

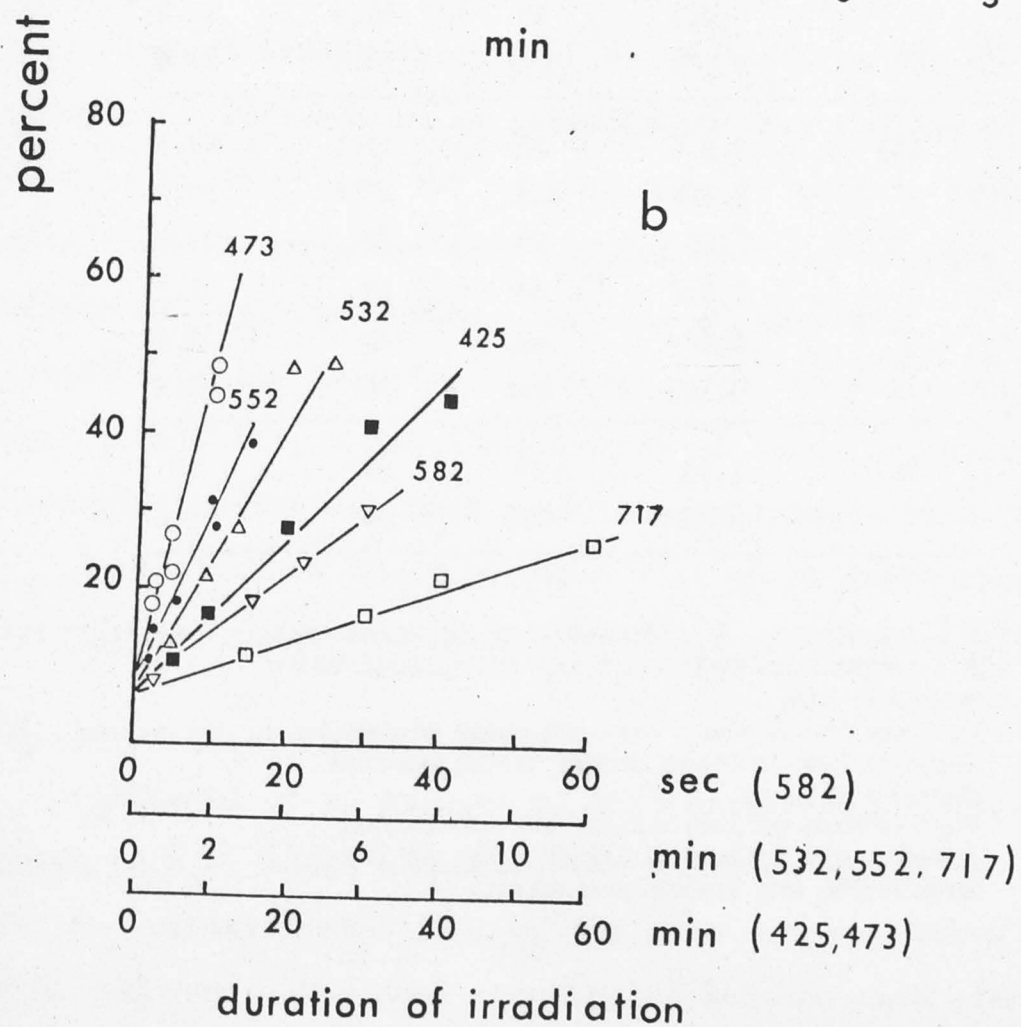
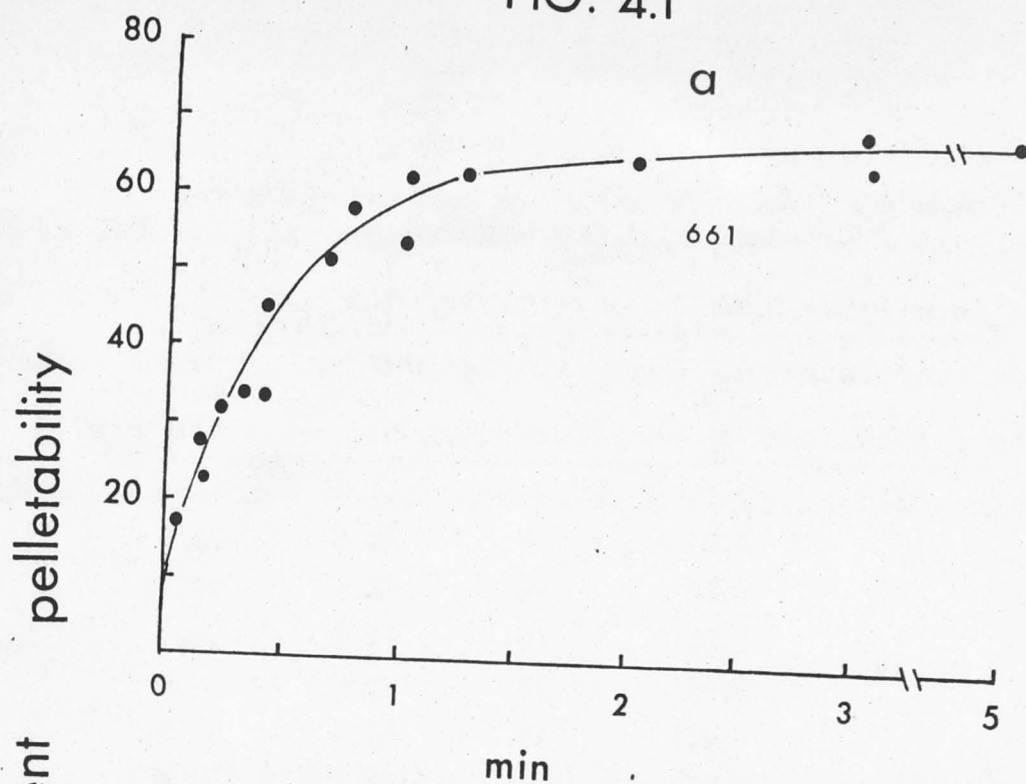


Table 4.1 Verification of the reciprocity law for short pulse irradiations

λ	I	t	I x t	B
nm	$\text{Jm}^{-2}\text{s}^{-1}$	s	Jm^{-2}	%
692	2.6	6	15.6	28
	1.3	12	15.6	30.8
	0.25	62	15.5	29.1
	1.95	10	19.5	38.7
	0.97	20	19.5	36.5
	0.487	40	19.5	36.8
	0.195	100	19.5	37.4
582	0.875	20	17.5	19.3
	0.115	150	17.2	18.3
	1.95	20	39	29.8
	0.975	40	39	31.4
	0.48	80	39	30.9
	0.195	200	39	32.8
552	1.15	60	69	19
	0.150	740	69	18.4

I : irradiance, t : duration of irradiation,
B : percentage pelletability calculated using
equation 3(8).

Various irradiances were obtained either by
varying lamp voltage and/or using neutral
density filters.

Phytochrome pelletability was determined
immediately following irradiation at the
wavelength and irradiance shown.

with an initial part which can be satisfactorily approximated to a straight line. For the purpose of constructing an action spectrum, the initial part of the curve was determined for each wavelength. Linear approximations to the initial parts of these curves are shown in Fig. 4.1b for some representative wavelengths.

Verification of the law of reciprocity for short pulse irradiation

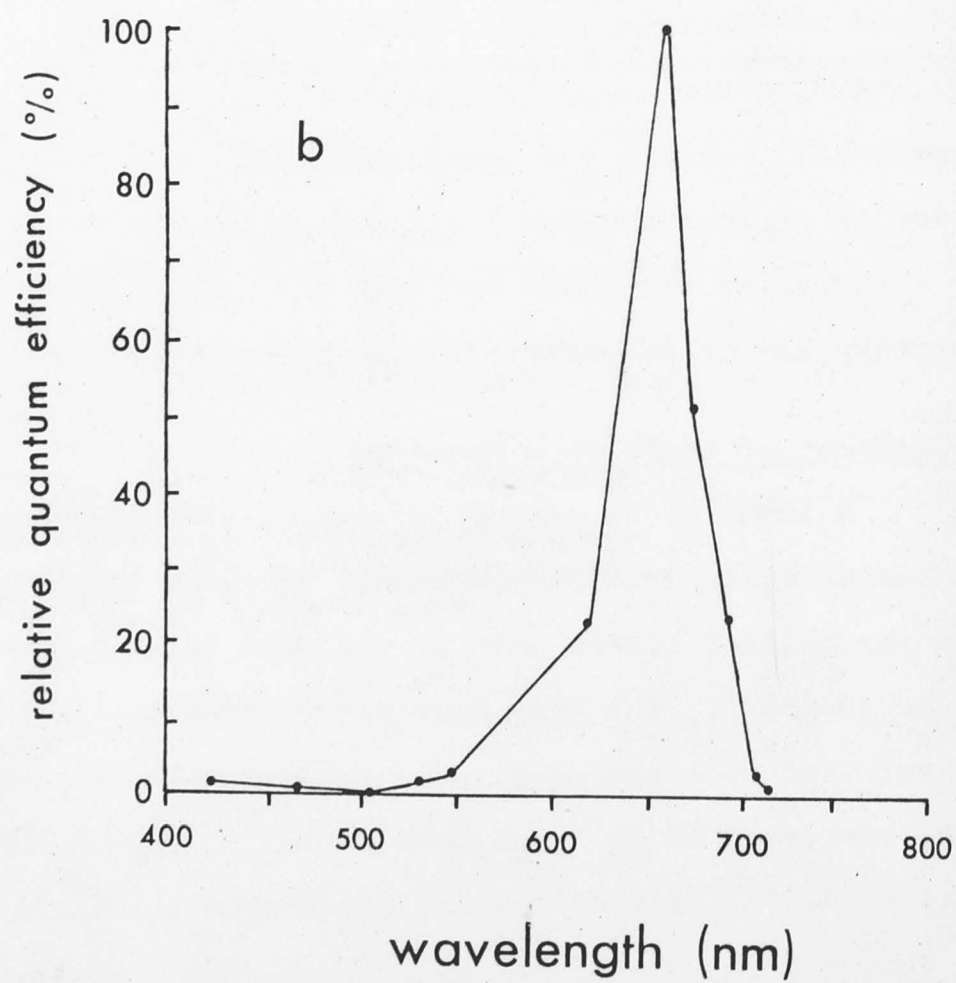
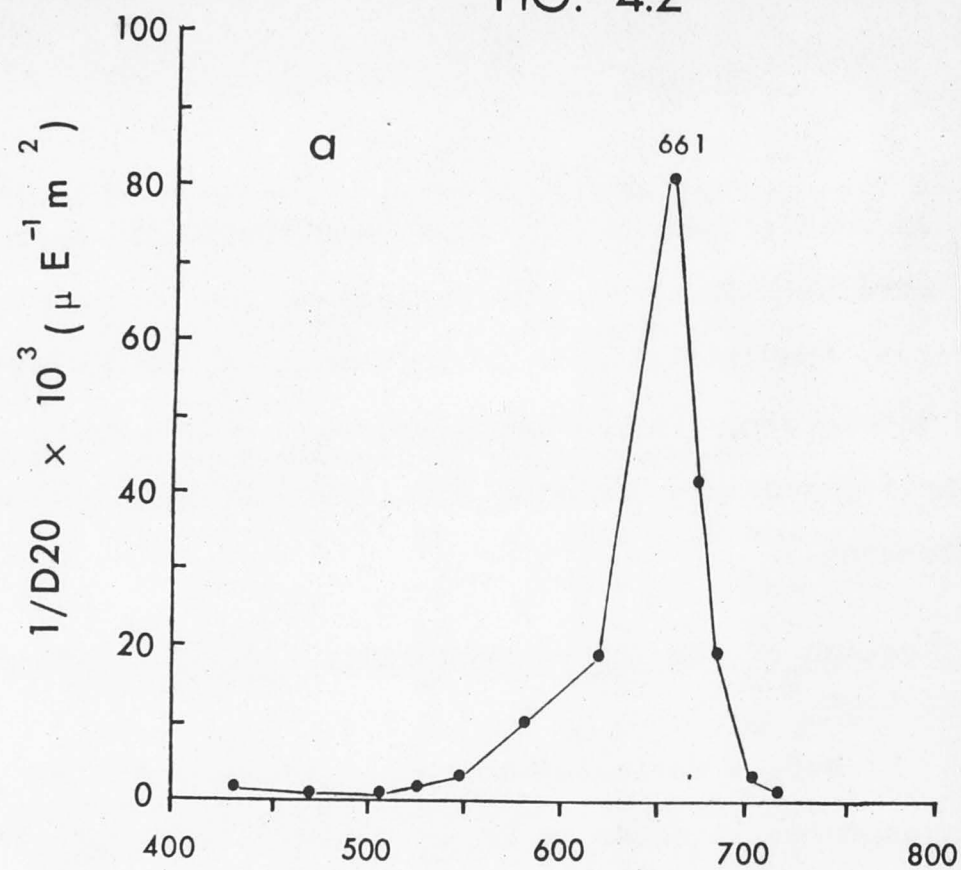
Before establishing the action spectrum for a photoresponse, it needs to be established that the law of reciprocity holds: i.e. for a given dose of actinic light, the extent of the response thus induced must be the same regardless of light intensity. Because an absolute verification of the law of reciprocity over the whole spectrum is impractical, only a few combinations of light intensity and duration of irradiation for 3 selected wavelengths were tested.

Results are shown in Table 4.1, which shows that the reciprocity law holds quite well for short pulse irradiation.

Establishment of the action spectrum

A level of 20 percent of phytochrome pelletability was taken as a standard photoresponse (in fact any value within the initial linear part of the dose response curve could be adopted). The dose required to produce this effect (D_{20}) was read from the dose response curve for each wavelength, expressed in Jm^{-2} and then in Em^{-2} . The reciprocal of this value is plotted against wavelength in Fig. 4.2a.

FIG. 4.2



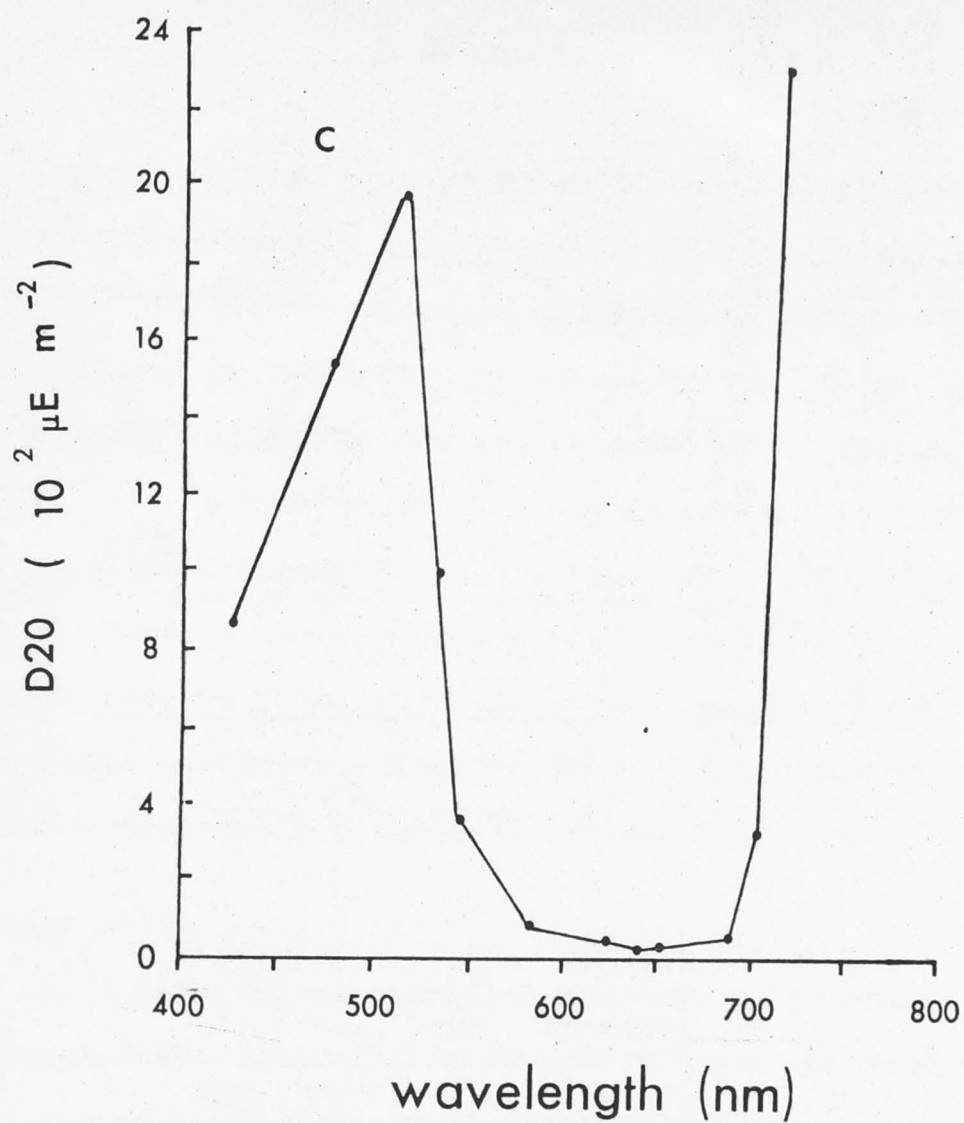


Fig. 4.2 Action spectrum for the induction of phytochrome pelletability *in vivo*.
D20: light dose required to induce 20% pelletability (see text for details).

FIG. 4.3

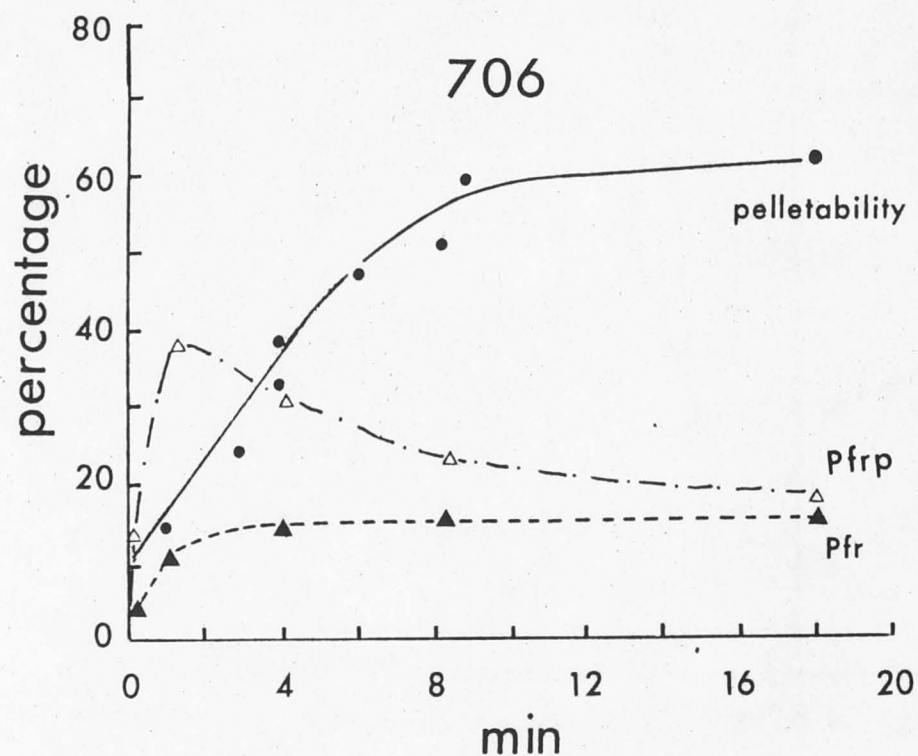


Fig. 4.3 Phytochrome pelletability and Pfr content following irradiation with light of 706nm. Phytochrome pelletability (●—●), percentage Pfr (▲---▲) and percentage Pfr in 30KP (△---△) as a function of duration of irradiation at 706nm.

The action spectrum is also shown in terms of relative quantum efficiency ($D_{20}^{661}/D_{20}^{\lambda}$) \times 100 (Fig. 4.2b).

The action spectrum generated by this procedure and depicted in these Figures shows the same major characteristics as the absorption spectrum of purified Pr (Fig. 1.1): a peak at about 660nm, a minimum near 500nm and a slight increase for $\lambda < 500\text{nm}$. This slight increase for $\lambda < 500\text{nm}$ becomes more apparent when the number of photons necessary to induce 20 percent pelletability (D_{20}) is plotted against wavelength; here the minimum of the action spectrum appears inverted as a maximum (Fig. 4.2c).

Maximum pelletability and photostationary state

Although only the linear portions of the dose response curves were necessary to construct the action spectrum of phytochrome pelletability, maximum pelletability and photostationary states were also determined for each wavelength. In Fig. 4.3, the dose response curve obtained for the induction of pelletability by light of wavelength 706nm is compared to that obtained for phototransformation; incidentally, the Pfr content measured in the pellet is shown. It is apparent that with this wavelength, saturation of pelletability (~60 percent) occurs for a light dose much greater than that necessary to establish the photostationary state (~15 percent Pfr), and the Pfr content of the pellet remains well below 40% before approaching the photostationary state value. In Fig. 4.4, maximum pelletability induced by

FIG. 4.4

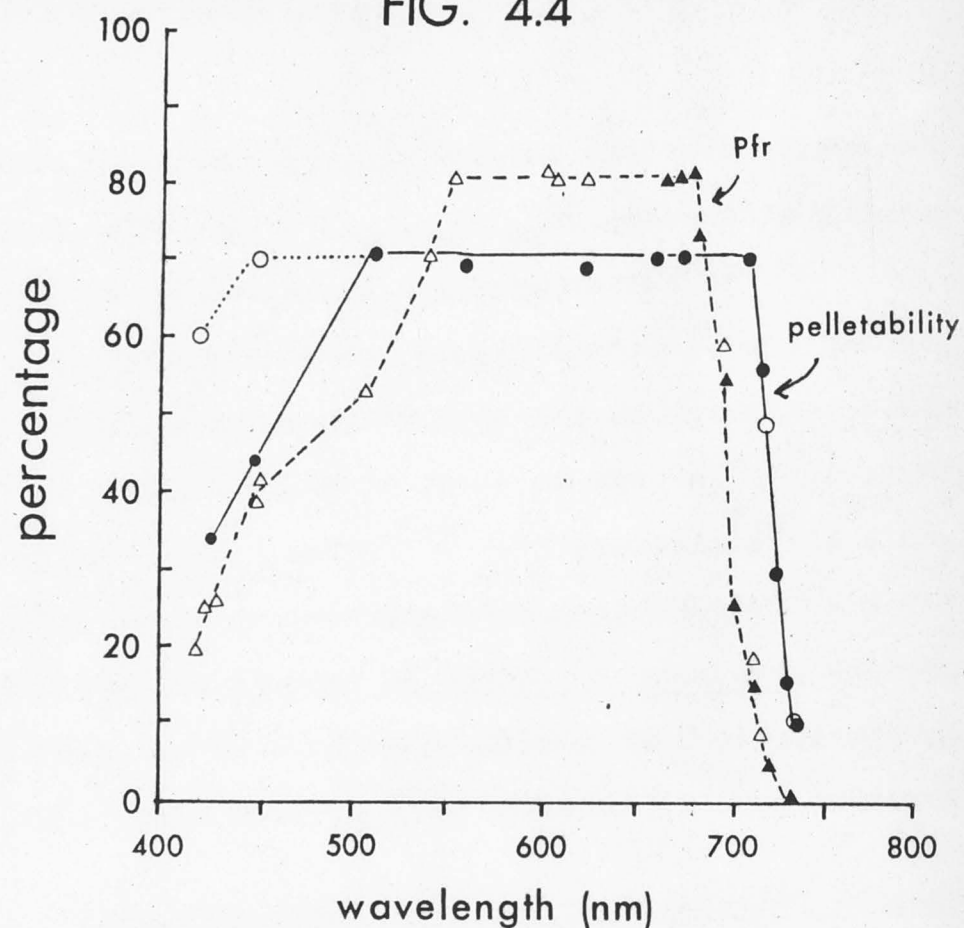


Fig. 4.4 Spectral dependence of maximum pelletability and photostationary states. Pfr was measured *in vivo* with filter B in the Ratiospect (\blacktriangle - \blacktriangle) or *in vitro* with filter A and corrected using equation 3(5) (\triangle - \triangle). (\bullet - \bullet) maximum pelletability obtained following 15-20 min of irradiation; (\circ - \circ) levels of pelletability obtained following 1 hour irradiation represented for the cases where saturation of phytochrome pelletability was not obtained with a 15-20 min irradiation.

irradiations with different wavelengths and photostationary states are plotted as a function of the wavelength of irradiation. Data presented in this Figure show that irradiation at any wavelength between 470nm and 720nm can induce a high level of pelletability similar to that induced by 660nm light, provided that a sufficient dose is given. Percentage Pfr however, remains well below the maximum value (80 percent) for wavelengths shorter than 550nm and longer than 680nm. The photostationary states determined are in good agreement with those reported by Hartmann and Spruit (1968, in Hanke *et al.* 1969).

Effect of sustained irradiation on phytochrome pelletability

It is well known that phytochrome disappears in red irradiated tissues during their subsequent incubation in the dark, by a process called destruction (see Chapter 1). Disappearance of phytochrome also occurs in tissues during continuous irradiation with red, blue and far-red light (Clarkson and Hillman 1967, Kendrick and Frankland 1968, Schäfer *et al.* 1975).

Although the destruction reaction will be investigated in more detail in Chapter 6, it is appropriate here to analyse the resultant influence of prolonged continuous irradiation on phytochrome pelletability when pelletability and destruction are allowed to proceed concurrently. Figs.4.5 and 4.6 show the results obtained after 4 h irradiation at room temperature. A 4 h irradiation is chosen so as to minimise

FIG. 4.5

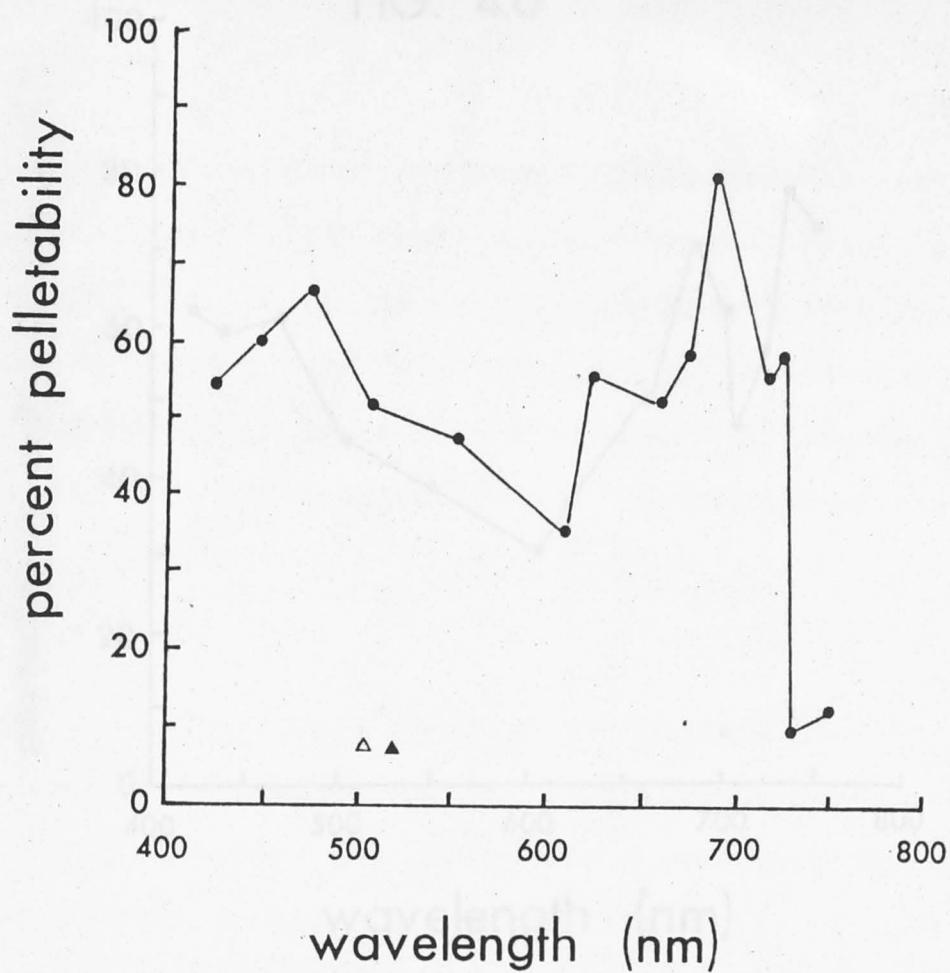


Fig. 4.5 Percentage pelletability obtained following 4 h of continuous irradiation, as a function of wavelength of irradiation. Pelletability was calculated using equation 3(8). Δ and \blacktriangle represent pelletability obtained following 4 h exposure to safelights C2 and B respectively.

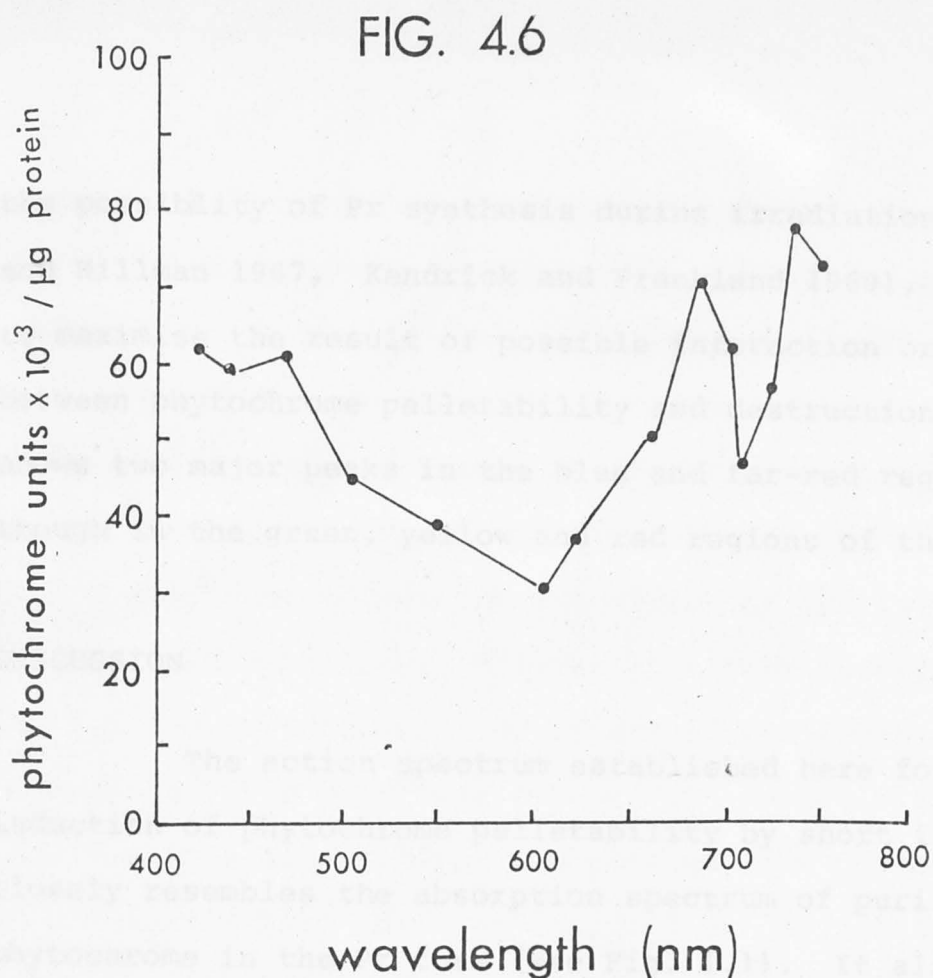


Fig. 4.6 Effect of 4 h continuous irradiation on total phytochrome content, as a function of wavelength of irradiation. Protein content was determined using the Lowry protein assay (Lowry 1951).

the possibility of Pr synthesis during irradiation (Clarkson and Hillman 1967, Kendrick and Frankland 1969), and also to maximise the result of possible interaction or competition between phytochrome pelletability and destruction. Fig. 4.5 shows two major peaks in the blue and far-red regions and a trough in the green, yellow and red regions of the spectrum.

DISCUSSION

The action spectrum established here for the induction of phytochrome pelletability by short irradiation closely resembles the absorption spectrum of purified phytochrome in the Pr form (see Fig. 1.1). It also resembles in part that of chlorophyll in solution (Goedheer 1966). Two facts argue however against the possibility that chlorophyll might be the primary photoreceptor : (i) the close resemblance between the absorption spectrum of Pr and the action spectrum here, especially in the blue region and (ii) the fact that the action spectrum was established for short irradiations on etiolated maize coleoptiles, presumably devoid of chlorophyll at the beginning of irradiation (see Boardman 1966). The well defined peak of the action spectrum at about 660nm also argues against protochlorophyll as a primary photoreceptor (see Koski *et al.* 1951). Phytochrome therefore is very likely the primary photoreceptor for its own association with a particulate fraction induced by short irradiation, at least in etiolated maize coleoptiles. Other

arguments in support of this statement are presented below.

Recently the induction of phytochrome pelletability was found to be a very fast reaction with a half-life at 0°C and 25°C of about 40 sec and 2 sec respectively (Pratt and Marmé 1976, Quail and Briggs 1978). Far-red light given within seconds of a short (5 sec) pulse of red irradiation at 0°C was found to reverse the effect of red light in inducing pelletability. However if a dark interval precedes the far-red irradiation, the effect of red light is no longer reversible. The very fast escape from reversibility of this reaction thus accounts for the general lack of immediate photoreversibility observed when 3-5 min of red light is given to induce pelletability (Quail *et al.* 1973a, Quail and Schäfer 1974, Table 3.3). Even in this case however, pelletability induced by 3-5 min red light is somewhat FR reversible: it was shown in Chapter 3 that phytochrome is slowly released from the bound condition during a subsequent dark incubation of R/FR or R irradiated coleoptiles at 0°C. The rate of release was found to be much slower following red irradiation only than following R/FR irradiation (Fig. 3.7). These observations (further discussed in Chapter 7) thus add support to the conclusion that phytochrome pelletability is a genuine phytochrome-mediated response as inferred by the action spectrum.

The dose response curves obtained for the establishment of the action spectrum indicate that for the range of

wavelengths studied, all wavelengths between 420nm and 720nm can induce pelletability, but with different efficiencies. Relatively larger doses of light (achieved here by increasing the duration of irradiation) are required for blue, green or far-red light, and are commensurate with the lower probability of light absorption at these wavelengths by phytochrome. Therefore, provided that sufficient light is absorbed by phytochrome, a high level of pelletability, similar to that induced by a short pulse of red light, can be obtained regardless of wavelength and photostationary state. It also follows that an irradiance dependency for pelletability should be expected for subsaturating doses of light as observed by Quail and Schäfer (1974). Incidentally, it may be appropriate here to redefine a saturating irradiation. Usually an irradiation is referred to as saturating if the light dose delivered is sufficient to establish the photostationary state characteristic of that light. It has become apparent however, that a light dose which is saturating as far as photoequilibrium is concerned, may not be so when one considers the pelletability of phytochrome (see Fig. 4.3). Therefore one may have to refer to 'saturating doses of phototransforming light' to emphasise these differences.

When relatively long irradiations are used to induce high pelletability by light establishing low photostationary states, the possibility of cycling of Pr to Pfr and Pfr to Pr

can be invoked to explain the association of Pr with the particulate fraction. Other explanations are however possible and will be the subject of Chapter 5.

The saturation of the pelletability capacity below 100 percent observed here, and also by other workers, suggests that the particulate receptor for phytochrome is limiting and that 20-30 percent of the total phytochrome remains soluble after saturating irradiations. This interpretation is in disagreement with the assumption (Schäfer 1975a, b, Schäfer *et al.* 1975, Lehman and Schäfer 1978) that the receptor for phytochrome is unlimited.

Although in short pulse irradiations, the destruction of phytochrome during irradiation may not interfere significantly with pelletability, its effect on pelletability during prolonged irradiation is quite remarkable.

Thus as shown in Fig. 4.6, phytochrome content was substantially reduced by prolonged irradiation (4 h). In terms of maintaining a high level of pelletability, far-red and blue lights appear to be more effective than red light under conditions of prolonged irradiation. The curves expressing percentage pelletability as a function of wavelength (Fig. 4.5) showed a resemblance to various 'action spectra' of responses induced under conditions of sustained irradiation, and also to the high irradiance response spectrum for the inhibition of lettuce hypocotyl lengthening (Hartmann 1967). Hartmann (1966) has interpreted the HIR responses as

resulting from two competing reactions: phytochrome destruction and phytochrome receptor interaction. Schäfer (1975c, d) developed the model of Hartmann further, via an open phytochrome-receptor interaction model. On the basis of theoretical calculations and data obtained on phytochrome destruction and synthesis, Schäfer derived a theoretical action spectrum for pelletability (PfrX) under HIR conditions. Only a rough comparison between that theoretical HIR action spectrum and the wavelength dependency of phytochrome pelletability obtained here can be made, because no irradiance dependency of the reaction (a characteristic of HIR, see Mohr 1964) has been investigated here. It remains that there is resemblance between the two spectra, so far as Schäfer's action spectrum predicts a peak in the far-red region (above 700nm) and at least one in the blue region (below 500nm) as observed here (Fig. 4.5).

If phytochrome pelletability can be shown to be directly related to physiological responses, then HIR responses would indeed be explained solely on the basis of phytochrome as envisaged by Hartmann (1966) and Schäfer (1975c, d).

CHAPTER 5

INTRODUCTION

In Chapter 4, the observation was made that irradiation of maize coleoptiles at a wavelength which establishes a low photostationary state can produce a high level of pelletability, similar to that induced by saturating red light. Examination of the state of phytochrome in the

CHAPTER 5

PHYTOCHROME PHOTOTRANSFORMATION

AND PELLETABILITY INDUCED BY

ULTRASHORT IRRADIATIONS

To explain the presence of excess P_r in the particulate fraction, the reaction models described below may be envisaged among various possible schemes.

In these models X represents the as yet unidentified receptor for phytochrome, which is pelletable upon fractionation of the tissue. For the sake of simplicity, the terms 'binding' and 'bind' are used formally throughout this chapter to designate the interaction of phytochrome with X , regardless of the nature of this interaction, i.e. whether it implies actual physical binding of phytochrome with X in vivo or is merely the induction in vivo of a potential for phytochrome to co-pellet with X . This question will be dealt with in detail in Chapter 7.

CHAPTER 5

INTRODUCTION

In Chapter 4, the observation was made that irradiation of maize coleoptiles at a wavelength which establishes a low photostationary state can produce a high level of pelletability, similar to that induced by saturating red light. Examination of the state of phytochrome in the pellet showed a high proportion of Pr. Pratt and Marmé (1976) also observed that 'at low Pfr levels, for each Pfr molecule produced by red light, one additional molecule of phytochrome (although some are Pr) is found in the pellet'.

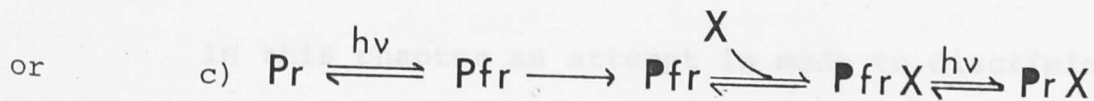
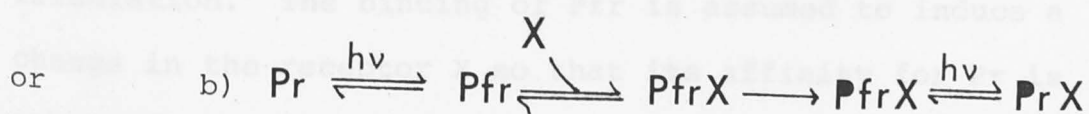
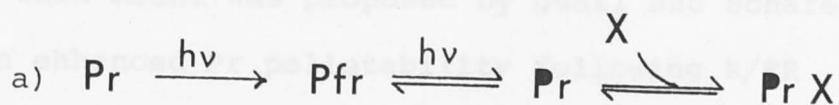
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Model 1:

This model accounts for the presence of both Pfr and Pr in the particulate fraction. Pr is first phototransformed to Pfr, binds to X in the Pfr form, and PrX results from the photoconversion of Pfr in the bound form. The companion model in which PrX originates from the dark reversion of PfrX has not been considered here because it is assumed that no dark reversion occurs in maize *in vivo* or in the sub-cellular fractions which are dealt with here (see results of Chapter 3).

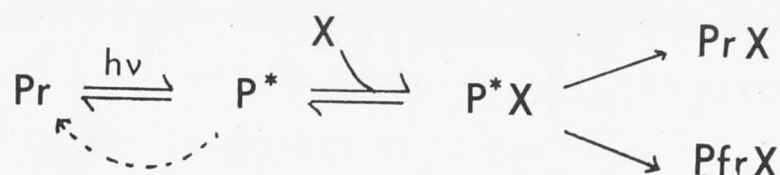
As alternatives to the photoconversion of phytochrome to Pr following the association of Pfr to X *in situ*, the following models may be proposed for the binding of Pr with X.

Model 2:

In this model the Pr that binds to X is unlike native Pr: it has been through the Pfr state at least once and retains a slight configurational change reminiscent of the Pfr state

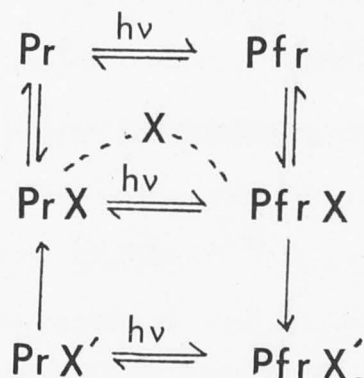
which (a) allows it to bind to X or (b, c) to remain associated with X.

Model 3:



Pr by absorbing light binds to X without necessarily being phototransformed to Pfr. P* in this case could represent one of the intermediates of the phototransformation in the Pr to Pfr pathway.

Model 4:



This model was proposed by Quail and Schäfer (1974) to explain enhanced Pr pelletability following R/FR irradiation. The binding of Pfr is assumed to induce a change in the receptor X so that its affinity for Pr is enhanced.

In this chapter an attempt is made to discriminate between these different models. In particular the hypothesis that Pr must go through the Pfr state at least once before associating with X is tested. I therefore investigated the induction of phytochrome pelletability by ultra short pulse irradiations (lms or less) that would not permit significant

FIG. 5.1

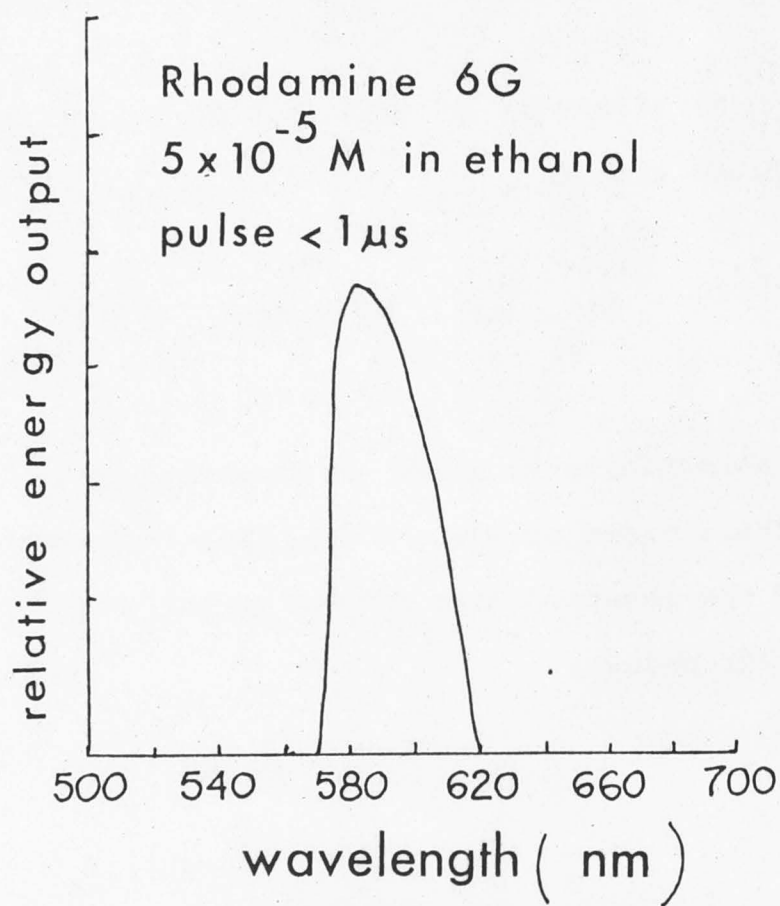


Fig. 5.1 Spectral distribution of the light pulse produced by the dye laser (Phase R, DL 1100), as specified by the manufacturers.

cycling of phytochrome between the Pr and Pfr states during the inductive irradiation (see below).

The experiments involved the irradiation of maize coleoptiles with a single laser pulse or a single flashlamp pulse, followed by the determination of Pfr content and pelletability.

EXPERIMENTAL

Laser irradiation

Laser pulses shorter than half a microsecond (μ s) (according to the manufacturers, Phase R company, New Durham, N.H.) were produced by a flashlamp-pumped dye laser (Phase R, DL1100). The laser was operated at 16 KV. The dye used was Rhodamine 6G, 5×10^{-5} M in ethanol. The beam diameter was 1 cm. Because of the short laser pulse duration, it was not possible to check accurately the spectral emission of the laser. The spectral characteristic as specified by the manufacturers for the dye concentration used is illustrated in Figure 5.1. It shows a band emission with a peak near 580 nm. The maximum energy output measured with a Scientech 362 power energy pulse radiometer was 140 mJ. Attempts to use other dyes (cresyl violet and oxazine) did not yield satisfactory lasing in the red or far-red regions.

Irradiation procedure

Because of the small beam size, excised coleoptiles were irradiated individually. For this purpose they were

mounted vertically on a disposable pipette tip, and only the free top end of the coleoptile placed in the path of the laser beam was recovered after irradiation for determinations of phytochrome pelletability and Pfr content.

Single pulse irradiation with a flashlamp

A camera electronic flash (Metz Mecablitz 502) in combination with an interference filter provided short pulse irradiations in the red region. The duration of the flash pulse measured to the $1/e$ decay point was 1.1ms (see Fig. 5.2a). Because of the relatively low intensity of the pulse (as compared to that of the laser) the beam was not collimated through the interference filter used. The filter was placed on the flashgun head, as close as possible to the flash tube. This has the effect of broadening the transmission band of the filter and of shifting the transmission peak 10nm towards the blue as illustrated in Figure 5.2b. The output maximum obtained through the red filter used (AL max 658nm, T_{max} 55%, HW 20.5nm) was approximately 50Jm^{-2} . Different light intensities were obtained using neutral density filters.

Irradiation procedure

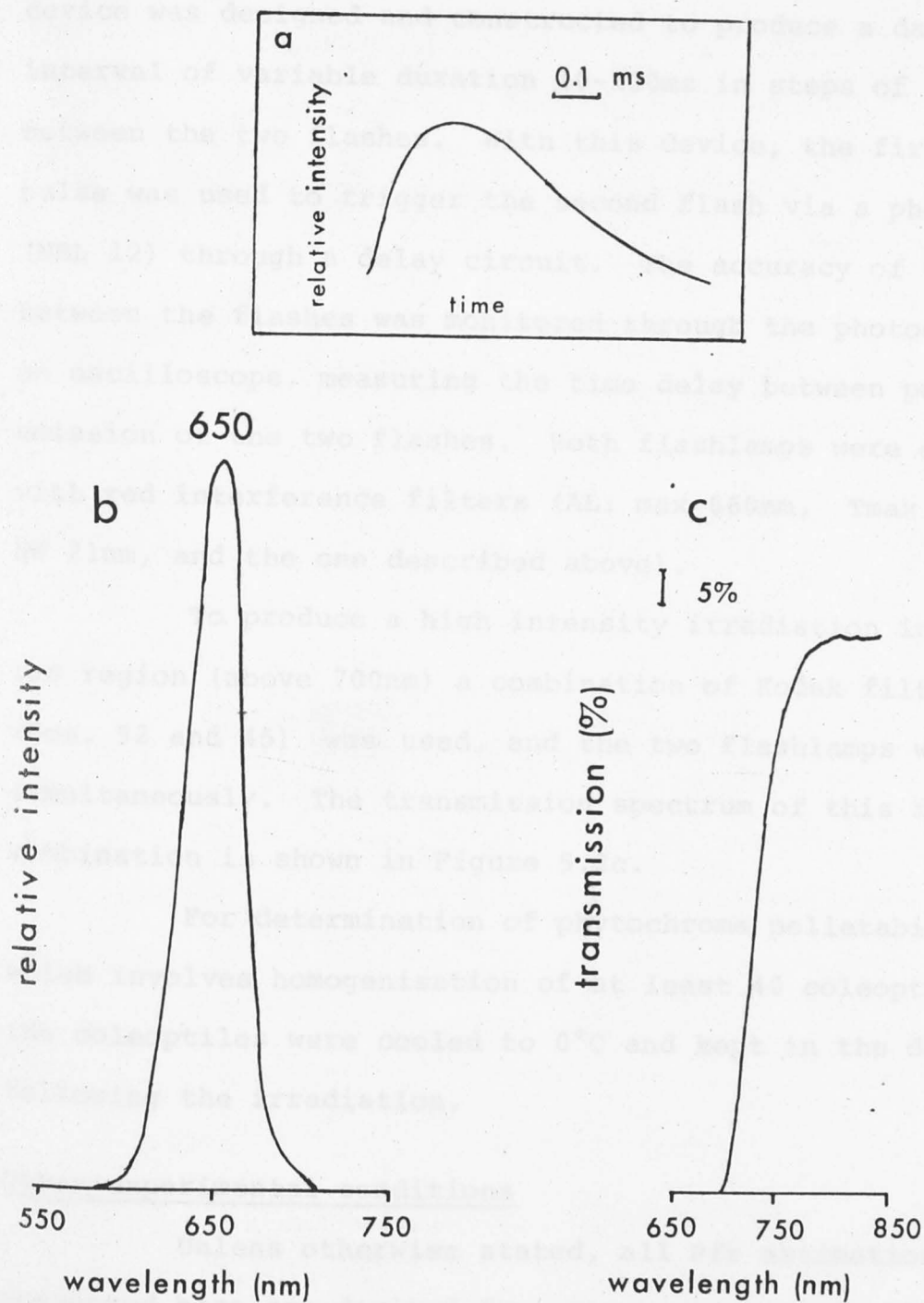
About ten coleoptiles (length < 1cm) were arranged on a microscope glass slide: this was placed just above the interference filter for irradiation.

Double flash irradiations

Another camera electronic flash (Metz Mecatwin 502) was used in conjunction with the one above to produce two

Fig. 5.2 a) time distribution of the flash pulse.
b) spectral distribution of the light obtained through an AL interference filter (λ max = 658nm) without collimation (see text for details), and analysed with an IL 780 spectroradiometer.
c) spectral distribution of the light obtained through a combination of colour filters (Kodak Nos. 92 and 45) and analysed in a Varian series spectrophotometer (model 6348).

FIG. 5.2



short pulses of light separated by a dark interval. A timing device was designed and constructed to produce a dark interval of variable duration (0-100ms in steps of 1ms) between the two flashes. With this device, the first light pulse was used to trigger the second flash via a photodiode (MEL 12) through a delay circuit. The accuracy of the delay between the flashes was monitored through the photodiode and an oscilloscope, measuring the time delay between peak emission of the two flashes. Both flashlamps were equipped with red interference filters (AL: max 660nm, T_{max} 64%, HW 21nm, and the one described above).

To produce a high intensity irradiation in the far-red region (above 700nm) a combination of Kodak filters (Nos. 92 and 45) was used, and the two flashlamps were fired simultaneously. The transmission spectrum of this filter combination is shown in Figure 5.2c.

For determination of phytochrome pelletability which involves homogenisation of at least 40 coleoptiles, the coleoptiles were cooled to 0°C and kept in the dark following the irradiation.

Other experimental conditions

Unless otherwise stated, all Pfr estimations presented here are derived from measurements made with filter B in place in the Ratiospect (see Chapters 2, 3). Type C safelights were used in double flash experiments and all experiments involving Pfr determinations *in vitro*. Pfr values

Table 5.1 Viability of coleoptiles following laser irradiation.

CURVATURE (deg.)		
	dark control	laser irradiated
n = 15	26.3	26.8
n = 17	28.3	22.5

Geotropic curvature developed
6 hours after treatment.
n = number of coleoptiles
(see text for details).

reported here were derived from *in vivo* measurements carried out in complete darkness following irradiation when only type B safelights were available (Fig. 5.4b). For all phytochrome measurements in the 30KP fraction, the pellet was resuspended in SEBM buffer.

RESULTS

Viability of coleoptiles following high intensity irradiation

To ascertain that the high light intensity pulse used in the experiments did not injure or kill the tissue, coleoptiles were tested for their response to gravity following irradiation at the highest light intensity used.

The excised coleoptiles were irradiated with a laser pulse of total energy 140mJ. The exposed part was cut to approximately 8mm. Each section thus received about 30mJ in less than 1 μ s. The tips were then placed horizontally with their bases in agar (1.5%). Curvature in response to gravity was allowed to develop for 6 h in darkness. The coleoptiles were shadowgraphed, and curvature measured and compared with that of dark controls. Results are shown in Table 5.1. The data shown are the results of two separate experiments. They show that the irradiated coleoptiles responded normally to gravity and are therefore presumed to have been physiologically uninjured.

FIG. 5.3

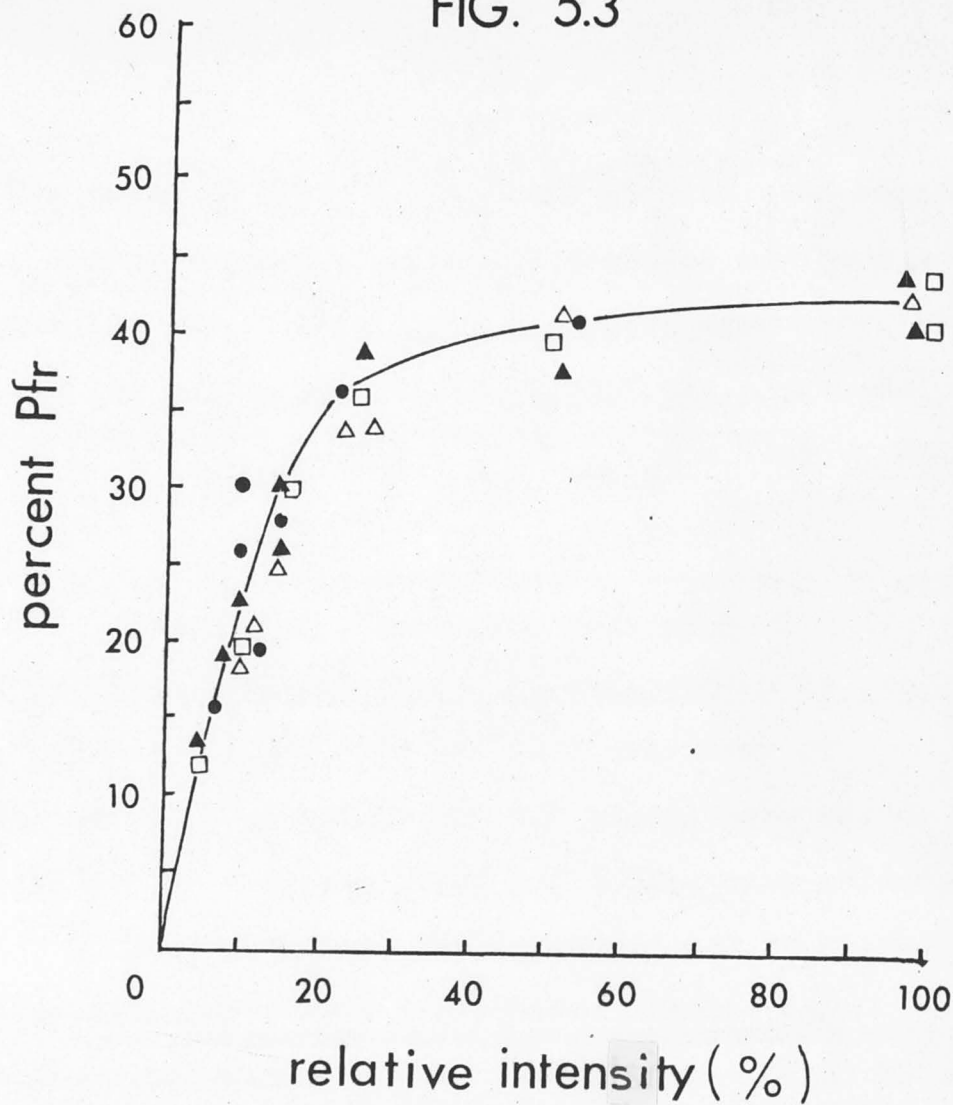


Fig. 5.3 Percentage Pfr following a single pulse irradiation as a function of light intensity. The various intensities are indicated as a percentage of the full intensity and were obtained using neutral density filters. Pfr measurements were made using filter B in the Ratiospect, except those represented by \square , which were made using filter A and corrected using equation 3(5).

\square : SL type B were in use

$\Delta, \triangle, \bullet$: SL type C were in use.

Single pulse irradiations

1. Dose response curve for phototransformation

To assess the efficiency of short light pulses in phototransforming Pr to Pfr, phytochrome phototransformation was assayed as a function of the light dose delivered by a single flashlamp pulse in the red (λ max : 650nm) region. The results are shown in Figure 5.3, and show that the phototransformation of Pr to Pfr increases as the light dose increases. A saturation level is however reached at which point 40-43% of phytochrome is transformed to Pfr.

2. Phototransformation vs. pelletability

The relationship between phototransformation and pelletability was then analysed. Phytochrome pelletability determined following flashlamp or laser irradiation is plotted as a function of Pfr content in Fig. 5.4a, b respectively. This figure shows that a laser pulse shorter than 1 μ s in duration at 580nm (λ max) induces phototransformation and pelletability in the same way as the longer (lms) flashlamp pulse at 650nm.

There is a linear positive relationship between pelletability and phototransformation. Levels of pelletability generally exceed those of Pfr content but the excess pelletability is generally comparable to the level of pelletability observed in dark controls.

FIG. 5.4

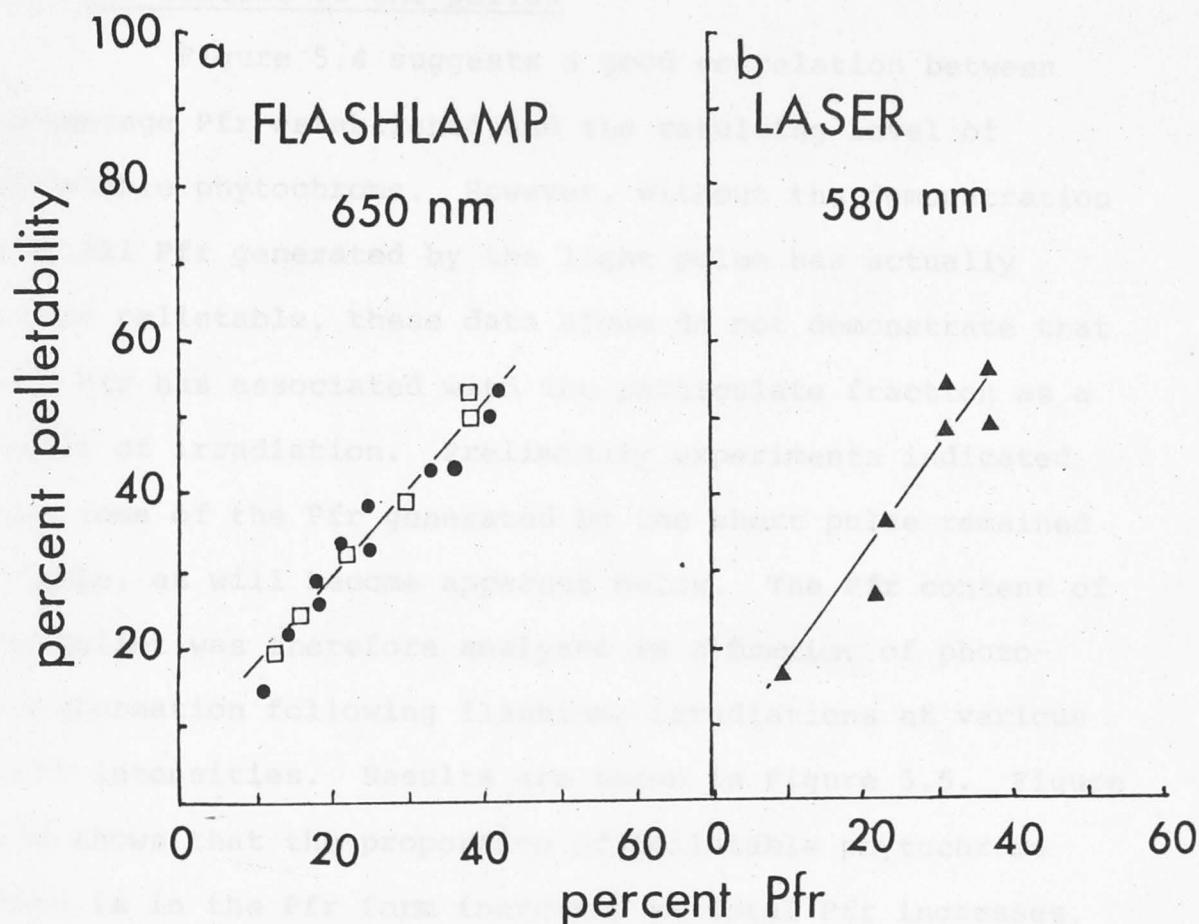


Fig. 5.4 Percentage pelletability obtained following a flashlamp (a) or laser (b) pulse, as a function of percent Pfr established. Pfr measurements were made using filter B in the Ratiospect, except those represented by \square , which were made using filter A and corrected using equation 3(5).
 \square, \blacktriangle : SL type B were in use;
 \bullet : SL type C were in use.
 Pelletability was calculated using equation 5(8).

3. Pfr content of the pellet

Figure 5.4 suggests a good correlation between percentage Pfr established and the resulting level of pelletable phytochrome. However, without the demonstration that all Pfr generated by the light pulse has actually become pelletable, these data alone do not demonstrate that only Pfr has associated with the particulate fraction as a result of irradiation. Preliminary experiments indicated that some of the Pfr generated by the short pulse remained soluble, as will become apparent below. The Pfr content of the pellet was therefore analysed as a function of photo-transformation following flashlamp irradiations at various light intensities. Results are shown in Figure 5.5. Figure 5.5a shows that the proportion of pelletable phytochrome which is in the Pfr form increases as total Pfr increases, but does not exceed 60%. The broken line shows calculations of the amounts which would be expected if only Pfr associates with X and takes into account also the contribution of pelleted phytochrome (Pr) usually present in dark controls. As shown here, when about 80% or more of the phytochrome in the pellet is expected to be in the Pfr form, only 60% was actually measured. The experimental curve is, on the other hand, close to the curve calculated on the assumption that for every two molecules of Pfr that bind to X, one additional molecule of Pr binds to X.

In Figure 5.5b the portion of the total phytochrome which is present as Pfr in the pellet (Pfr_p) is plotted as a

Fig. 5.5 Pfr content of the 30KP, as a function of the total Pfr content following a light pulse

a) Proportion of the phytochrome in the 30KP which is in the Pfr form:

●—● experimental points, ▲—▲ values calculated (from the data of Fig. 5.4a) on the assumption that only Pfr binds, or that for every two molecules that bind to X, one additional molecule of Pr binds to X ▲---▲.

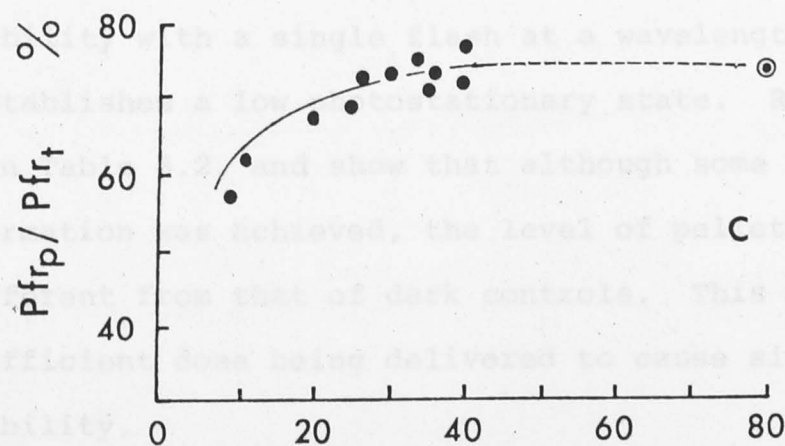
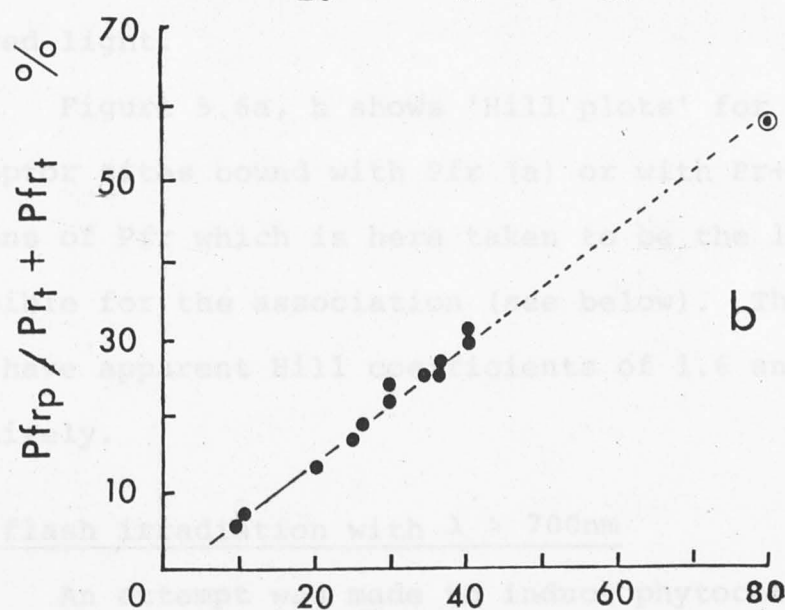
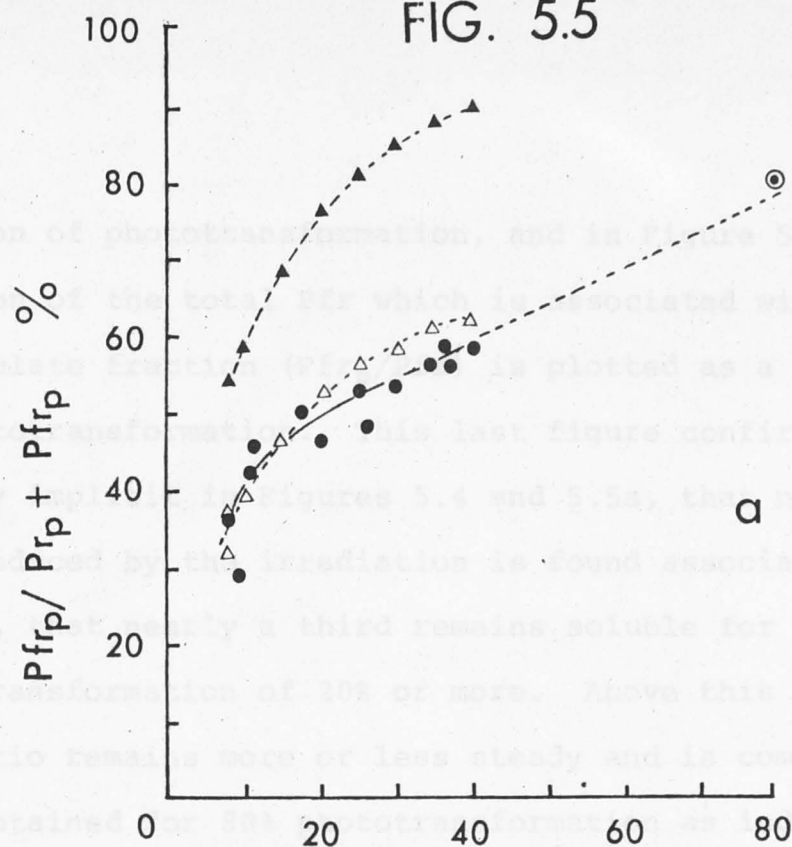
b) Portion of the total phytochrome ($Pr_t + Pfr_t$) which is present as Pfr in the pellet (Pfr_p).

c) Portion of the total Pfr (Pfr_t) which is present in the pellet.

⊙ values obtained following 5 min of continuous irradiation with red light (661nm).

30KP fractions were resuspended in SEBM, Pfr measurements were carried out with filter B in the Ratiospect. The safe lights used were of type C.
(see text for details)

FIG. 5.5



percentage Pfr

function of phototransformation, and in Figure 5.5c the fraction of the total Pfr which is associated with the particulate fraction (Pfr_p/Pfr) is plotted as a function of phototransformation. This last figure confirms what is already implicit in Figures 5.4 and 5.5a, that not all of the Pfr produced by the irradiation is found associated with X: rather, that nearly a third remains soluble for a level of phototransformation of 20% or more. Above this Pfr level the ratio remains more or less steady and is comparable to that obtained for 80% phototransformation as induced by 5 min red light.

Figure 5.6a, b shows 'Hill plots' for the number of receptor sites bound with Pfr (a) or with Pr+Pfr (b) as functions of Pfr which is here taken to be the ligand responsible for the association (see below). These 'Hill plots' have apparent Hill coefficients of 1.6 and 2.1 respectively.

Single flash irradiation with $\lambda > 700nm$

An attempt was made to induce phytochrome pelletability with a single flash at a wavelength ($\lambda > 700nm$) that establishes a low photostationary state. Results are shown in Table 5.2, and show that although some phototransformation was achieved, the level of pelletability was not different from that of dark controls. This may be due to insufficient dose being delivered to cause significant pelletability.

Fig. 5.6 Hill plots for phytochrome pelletability as a function of phototransformation, from the data of Figs. 5.4a and 5.5.

Pfr_p is the amount of Pfr present in pellet, normalised to 100 units of total phytochrome.

$Pfr_p \text{ max}$: value obtained following 5 min continuous irradiation with red light.

B = amount of total phytochrome present in the pellet normalised to 100 units of total phytochrome ($Pr_p + Pfr_p$).

B_{max} = value obtained following 5 min continuous irradiation with red light.

B_0 = dark control value.

FIG. 5.6

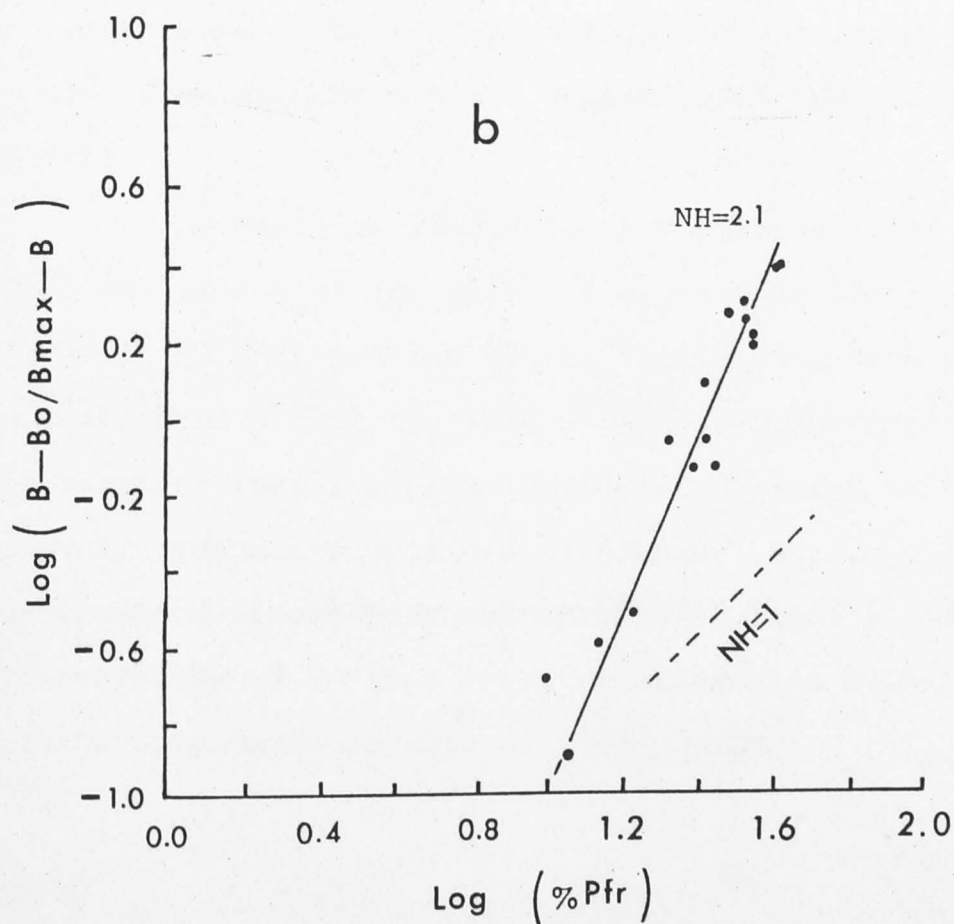
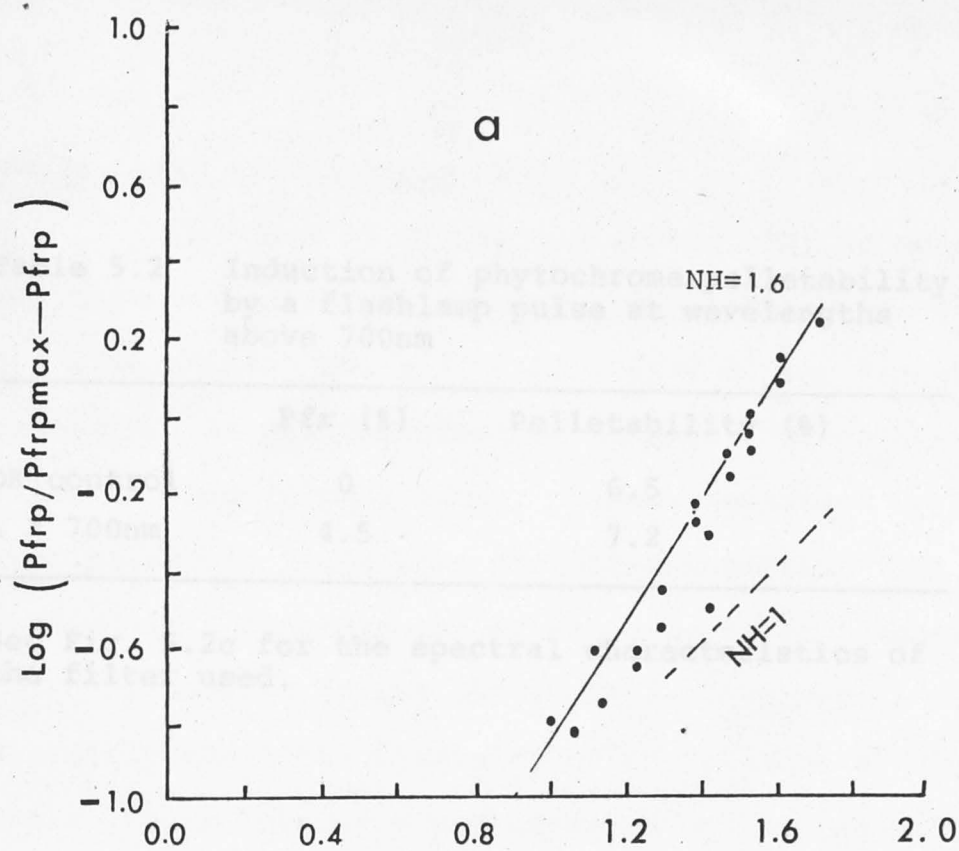


Table 5.2 Induction of phytochrome pelletability by a flashlamp pulse at wavelengths above 700nm

	Pfr (%)	Pelletability (%)
DK control	0	6.5
$\lambda > 700\text{nm}$	4.5	7.2

See Fig. 5.2c for the spectral characteristics of the filter used.

Double flash irradiations

The data of Figure 5.3 showed that no more than 43% of phototransformation of Pr to Pfr could be achieved in a single flash pulse (1ms or $< 1\mu\text{s}$) of the highest intensity. It was therefore of interest to see whether or not two red (λ max: 650nm) pulses, separated by a dark interval, would yield more phototransformation than that achieved in a single pulse of the same total intensity.

Because of the difficulties involved in measuring accurately the light intensity of the flashes at the sample levels, their intensity was adjusted so that each flash used independently would induce the same level of phototransformation for subsaturating doses of phototransforming light (i.e. for example, when used with a 10% transmission neutral density filter).

The phototransformation and pelletability achieved by the two pulses of red light is plotted in Figure 5.7a as a function of the duration of the intervening dark period. The results show that the levels of phototransformation and pelletability increase with increasing duration of dark interval. The curve obtained is complex, but to a first approximation it can be resolved into at least 2 components representative of 2 first order kinetics with half-lives of approximately 26ms and shorter (Fig. 5.7b).

Fig. 5.7 Double flash irradiations.

a) the percentage of phototransformation (Δ - Δ) and the percentage of pelletability (\blacktriangle - \blacktriangle) obtained following 2 flash pulses separated by a dark interval are plotted as a function of the duration of the dark interval.

b) reduction of the Pfr curve obtained in Fig. 5.7a. The points plotted are taken from the curve drawn in Fig. 5.7a (Pfr) and are not all experimental points.

Pfr₀, Pfr max, Pfr_t are % Pfr obtained when the dark interval between the light pulses were 0 ms, 100 ms, t ms respectively.

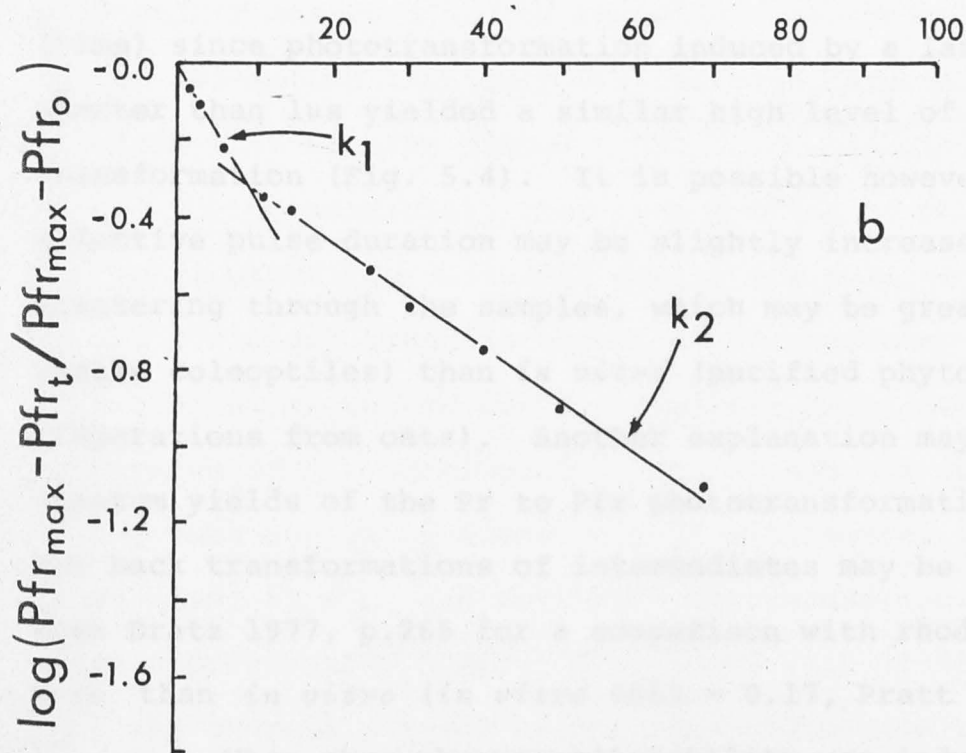
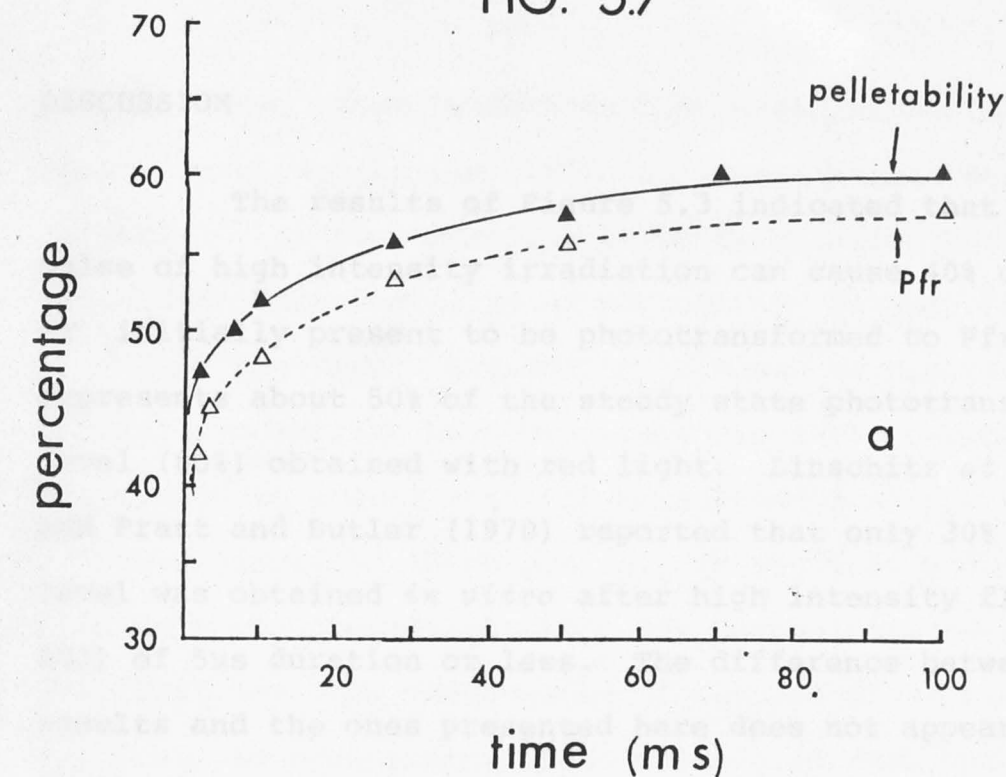
30KP was resuspended in SEBM.

Pelletability calculated using equation 3(7).

Pfr measurements were made with filter B in the Ratiospect, SL were type C.

log(Pfr - Pfr / Pfr - Pfr)

FIG. 5.7



DISCUSSION

The results of Figure 5.3 indicated that a single pulse of high intensity irradiation can cause 40% of the Pr initially present to be phototransformed to Pfr. This represents about 50% of the steady state phototransformation level (80%) obtained with red light. Linschitz *et al.* (1966) and Pratt and Butler (1970) reported that only 30% of this level was obtained *in vitro* after high intensity flash (400J, 80J) of 5 μ s duration or less. The difference between their results and the ones presented here does not appear to be simply due to the longer pulse provided by the flashlamp (\sim lms) since phototransformation induced by a laser pulse shorter than 1 μ s yielded a similar high level of phototransformation (Fig. 5.4). It is possible however that the effective pulse duration may be slightly increased by light scattering through the samples, which may be greater *in vivo* (maize coleoptiles) than *in vitro* (purified phytochrome preparations from oats). Another explanation may be that the quantum yields of the Pr to Pfr phototransformation and of the back transformations of intermediates may be different (see Dratz 1977, p.265 for a comparison with rhodopsin) *in vivo* than *in vitro* (*in vitro* $\Phi_{665} = 0.17$, Pratt 1975a).

When phytochrome pelletability was induced with a single light pulse, the proportion of phytochrome in the Pr form in the pellet was in excess of that expected if only Pfr could bind to X as a result of irradiation (Fig. 5.5).

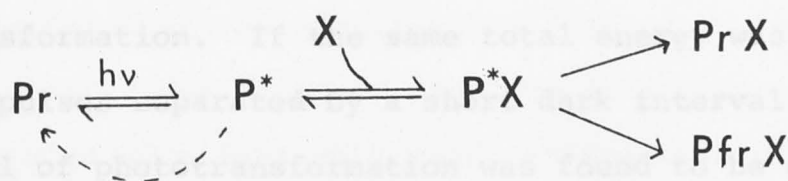
Two assumptions have been made in the interpretation of the results: i) the Pfr measurements in the pellet are reliable, ii) the flashlamp pulse irradiation does not cause significant cycling of phytochrome between Pr and Pfr.

The first assumption is based on the fact that careful precautions have been taken in the experiments described, to ensure that Pfr measurements were free of the problems described in Chapter 3. It could be argued that despite these precautions, the Pfr determinations are still imperfect and that improvement of methods might yield different results. In refutation, it is considered that the differences between Pfr measured in the pellet and the value anticipated on the assumption that only Pfr can bind, are large enough to conclude that Pr also binds.

The possibility that some cycling of phytochrome from Pfr to Pr might have occurred during the millisecond duration of the flashlamp irradiation was also considered. Linschitz *et al.* (1966) showed in flash photolysis experiments that the appearance of Pfr (monitored as an increase of absorbancy at 724nm) does not begin almost a full millisecond following the flash (5 μ s) at 0°C, and that full phototransformation to Pfr takes approximately 5 sec. Briggs and Fork (1969a) claimed that the overall process of phototransformation is temperature independent between 10°C and 20°C. Although the rate of phototransformation may be higher at 25°C than at 0°C, it is very unlikely that the amount of conversion of Pfr to Pr, sufficient

to account for the results, would have occurred during the short pulse irradiation used here (1 ms). It could be argued that, because of the exponential decay of the flash pulse, light actually remains well after 1 ms. However, the light intensity during that time would be too low to cause significant Pfr to Pr conversion. Therefore it is considered that the single flashlamp pulse does not cause cycling between Pr and Pfr, although cycling between Pr and an intermediate of phototransformation (lumi R or Meta Ra [Kendrick and Spruit 1977] can not be excluded (Pratt and Butler 1970).

From this it follows, in the case of flashlamp pulse irradiation, that the enhanced Pr pelletability observed in experiments described in this chapter cannot be explained by models 1, 2 or 4, since they all imply that phytochrome must be driven by irradiation into and out of the Pfr state at least once before binding to X. We are therefore left with model 3.



Validity of this model would require there to be at least one intermediate of phototransformation in the Pr to Pfr pathway, which is sufficiently long-lived to permit its interaction with X and which can also relax to Pr or to

Pfr. There are indications that the first requirement can be met. Some intermediates of phototransformation have been shown to be long-lived *in vitro*, having half-lives ranging from about 0.2ms to 2 sec at 0°C (Linschitz *et al.* 1966, Pratt and Butler 1970). Long-lived intermediates of phototransformation have also been shown to accumulate *in vivo* at 0°C and 25°C under conditions of pigment cycling (Briggs and Fork 1969a, b, Kendrick and Spruit 1972). Half-lives of 4.5 sec (Meta Ra) and 36 sec (Meta Rb) have been reported *in vivo* for intermediates of phototransformation at -11°C (Kendrick and Spruit 1973a). The possibility that some intermediates may be active, as first envisaged by Hillman (1967), cannot therefore be rejected *a priori*. The results of double flash experiments in the present study may provide further support for the existence of long-lived intermediates *in vivo* in maize, at physiological temperatures, as discussed below.

Single pulse irradiation yielded about 40% phototransformation. If the same total energy was delivered in two pulses separated by a short dark interval (100ms), the level of phototransformation was found to be enhanced by approximately 50%. This enhancement is a function of the duration of the dark interval between the two light pulses and appears to be limited by dark reactions showing complex kinetics. In a first approximation, these kinetics could be resolved into at least 2 reactions with half-lives of

approximately 26ms and shorter. A possible interpretation of the results is given below.

The two components of the kinetic can be interpreted to represent either (i) the photoconversion to Pr by the second flash, of intermediates of phototransformation which decay with half-lives of 8ms and 26ms; and/or (ii) dark relaxations to Pr (ground state) of intermediates of phototransformation, with half-lives of 8ms and 26ms. Both reactions would obviously limit the amount of Pr that can be phototransformed to Pfr by the second flash. Haupt and Scheuerlein (1977) have favoured the second alternative in interpreting the results of double flash experiments on seed germination.

Another explanation of the data could be that suggested by Haupt and Bretz (1976). These authors studied the chloroplast movement in *Mougeotia* as induced by flashes of polarised red light. They observed that two flashes separated by a dark interval of a few (30) ms are much more effective than two simultaneous flashes in inducing the response. They proposed that two light reactions in series separated by a fast dark reaction are involved in the Pr to Pfr pathway of phototransformation.

Whatever the true mechanisms involved may be, the data obtained (Fig. 5.6) are consistent with the existence of long-lived intermediates of phototransformation in maize at physiological temperature. The question of whether or not

these intermediates are sufficiently long-lived to permit binding remains. As a corollary to model 3, the rate of the binding reaction between P^* and X must be at least of the same order of magnitude as the rate of the intermediate decay. If the postulated binding of an intermediate of phototransformation with X occurs at a rate comparable to that observed for Pfr in maize and oats, i.e. with a half-life of 2 sec at 25°C (Quail and Briggs 1978), then the intermediates of phototransformation observed here are not good candidates for the postulated P^* ; a conclusion supported by the parallelism observed between the pelletability and the phototransformation curves of Fig. 5.6. The validity of model 3 cannot therefore be fully established here.

Recently however, Rüdiger (1978) proposed a scheme for the phototransformation pathways of phytochrome which is a modification of that proposed by Kendrick and Spruit (1977) (Fig. 1.2). Rüdiger's scheme allows for: (i) intermediates of phototransformation with even longer half-lives than those already mentioned (order of min, even hours), and (ii) the relaxation to Pr or Pfr of all intermediates in the Pr to Pfr pathway. This would fulfill all requirements for the acceptance of model 3.

Alternative speculative interpretations for the conclusion reached in this chapter that Pr can bind to X, without being phototransformed to Pfr, are briefly outlined below.

It can be envisaged that (i) the binding of Pfr to X induces the binding of native Pr (possibly by a Pfr induced change of the receptor X), or (ii) that *in vivo*, the phytochrome molecule contains more than one chromophore (e.g. phytochrome could exist *in vivo* as a polymer of the 120000d photoreversible unit usually obtained following its purification (Rice and Briggs 1973) and that phototransformation of one subunit, but not necessarily all, may be sufficient to induce binding of the polymer.

The relevance of models 1, 2 and 4 in explaining enhanced Pr pelletability under conditions of continuous irradiation (i.e. when cycling of phytochrome is allowed to proceed), is examined below.

Lehman and Schäfer (1978) have presented arguments in support of model 4. These are based on kinetic data of phytochrome pelletability obtained by carefully timing a dark interval (order of secs) between a pulse of red light (5 sec) and far-red (10 sec) irradiation at 0°C *in vivo*. This model which was introduced by Quail and Schäfer (1974) is based on a theoretical model of cooperative behaviour of proteins: the 'induced fit theory' of Koshland and Neet (1968). It has been favoured by Schäfer (1975a) on the basis of flashlamp irradiation experiments analogous to those presented here.

The data presented here differ from those of Schäfer (1975a) in several respects which may be accounted for by the different methods of fractionation used in the two

studies. The fractionation procedure adopted by Schäfer is a long one and the pellet was resuspended in Mg^{++} -free buffer. It is possible therefore that the data of Schäfer may suffer from the inaccuracies in the determination of phytochrome pelletability and Pfr levels discussed in Chapter 3, which may lead to an underestimation of phytochrome pelletability. Indeed the data of Schäfer indicate that for 80% phototransformation (presumably obtained following saturating continuous red light) only 50% pelletability was obtained, while recent pelletability values using faster fractionation procedures are higher, usually 60-70% in maize (Pratt and Marmé 1976, Quail and Briggs 1978, Chapter 4, Figs. 4.1a and 4.4).

The results of experiments described in this chapter showed apparent linear relationships between percentage Pfr, established by a pulse of red light, and both pelletable Pfr (Pfr_p) and total pelletable phytochrome ($Pr_p + Pfr_p$). Unlike that in Schäfer's report, no sigmoidal shape curves were observed and therefore, on this ground alone, there was no sign of the cooperativity claimed by Schäfer. Pratt and Marmé (1976), who also observed an excess of association of Pr with the particulate fraction (which can not be 'photochemically derived from previously bound Pfr' cf. model 1), did not find signs of cooperativity either.

From a plot of Pfr_p/Pfr vs. Pfr, Schäfer concluded that 'the binding is cooperative and the availability of the receptor sites is unlimited'. Quite a different behaviour of Pfr_p/Pfr vs. Pfr was found here: above 20% phototransformation,

the ratio did not vary much and was similar to that obtained following 5 min of continuous red light (Fig. 5.5).

The fact that 'Hill coefficients' calculated here are in agreement with those reported by Schäfer, and differ from unity, does not necessarily imply cooperativity in the binding. It may indeed be premature to transpose and apply the theory underlying the construction of Hill plots to phytochrome pelletability. A meaningful interpretation of Hill plots relies on a number of assumptions regarding the ligand-receptor system concerned, which are not well defined in the phytochrome pelletability (Quail 1978b) reaction (e.g. that the receptor is a dimer with two receptor sites, that there is physical association of the ligand with the receptor X, and that the intrinsic association constants for binding at the first site is much higher than that at the second site: none of these assumptions have been clearly verified).

In this chapter therefore, no evidence was found to support model 4. However, the possibility must be entertained that, despite the considerations discussed above, this model might be found to describe satisfactorily all the data recently obtained from kinetic studies of phytochrome pelletability (Pratt and Marmé 1976, Quail 1978a, Quail and Briggs 1978, Lehman and Schäfer 1978). In my view, acceptance of this model would necessarily imply the association of phytochrome with X *in vivo*, rather than an artifactual association

following a disruption of the tissue, as proposed by Quail (1978a, b).

Under conditions of continuous irradiation, cycling occurs and it is therefore not possible immediately to exclude model 1. Model 1 however implies that Pr is photochemically derived from PfrX, which implies that phytochrome associates with X during irradiation, i.e. *in vivo*.

Model 2 on the other hand, can be considered to be consistent with the hypothesis that the actual association of phytochrome with the particulate partner might occur during homogenisation of the tissue following irradiation. This model requires the existence of a configurational modification of phytochrome following transformation to Pfr which will be further discussed in Chapter 7.

CHAPTER 6

INTRODUCTION

Following irradiation of etiolated seedlings, phytochrome undergoes reactions in vivo which include reversion and destruction in dicotyledons and destruction in monocotyledons (see Chapter 1). Destruction of phytochrome implies loss of its stability and hence of its activity.

CHAPTER 6

PHYTOCHROME DESTRUCTION IN
RELATION TO PHOTOTRANSFORMATION
AND PELLETABILITY

1973c, Pratt et al. 1974). It has been reported to be dependent on metabolic activity, being virtually prevented at 0°C (Gruenewald and Hillman 1961; also inhibited by metabolic inhibitors (Butler and Lane 1965); while Gruenewald et al. (1965) proposed that it is a metal-dependent process, possibly oxidative, but not directly linked to respiration. Phytochrome destruction has been shown in some cases to be directly related to certain morphogenic responses (Smith and Attridge 1970, Galze-Karcow and Mohr 1973, Galze-Karcow et al. 1976).

It is generally believed that the Pr form of phytochrome is stable, whereas the Pfr form is specifically

CHAPTER 6

INTRODUCTION

Following irradiation of etiolated seedlings, phytochrome undergoes reactions *in vivo* which include reversion and destruction in dicotyledons and destruction in monocotyledons (see Chapter 1). Destruction of phytochrome implies loss of photoreversibility and hence of detectability. Little is known about the mechanistic basis and the physiological significance of the process of destruction. There is evidence that it may involve a proteolytic degradation of the chromoprotein (Quail *et al.* 1973c, Pratt *et al.* 1974). It has been reported to be dependent on metabolic activity, being virtually prevented at 0°C (Furuya and Hillman 1964); and also inhibited by metabolic inhibitors (Butler and Lane 1965); while Furuya *et al.* (1965) proposed that it is a metal-dependent process, possibly oxidative, but not directly linked to respiration. Phytochrome destruction has been held in some cases to be directly related to certain morphogenic responses (Smith and Attridge 1970, Oelze-Karow and Mohr 1973, Oelze-Karow *et al.* 1976).

It is generally believed that the Pr form of phytochrome is stable, whereas the Pfr form is specifically

susceptible to destruction (Hillman 1967, Mohr 1972, Smith 1975).

In dicotyledons, under conditions of continuous irradiation, the rate of phytochrome destruction is proportional to the photostationary state established (see Frankland 1972). By contrast, results obtained with monocotyledons indicate that destruction is independent of photostationary state over a wide range, and that destruction also appears to be saturated at low photostationary states under continuous irradiation (see Schäfer *et al.* 1975 for a list of references).

Schäfer *et al.* (1975, 1976) speculated that the rapid binding of Pfr to a receptor is an intermediary dark reaction between phototransformation of phytochrome to Pfr and its subsequent destruction.

The results described in the previous chapters suggested that Pr as well as Pfr is able to become associated with a particulate fraction in irradiated coleoptiles of maize. In view of Schäfer's speculation, an investigation of the possible involvement of Pr in the dark destruction of phytochrome was undertaken. Such a possibility has been considered also by Chorney and Gordon (1966) and Dooksin and Mancinelli (1968). Relationships between phytochrome pelletability, phototransformation of phytochrome into Pfr and destruction of phytochrome in the dark will be examined here as a function of wavelength of inductive irradiation in

the hope of clarifying these speculations.

Boisard *et al.* (1974) reported that, following red light treatment, phytochrome destruction is related exclusively to the phytochrome fraction which becomes pelletable as a result of the red irradiation. They concluded that the loss of photoreversibility of Pfr occurs at some membrane with which the phytochrome is believed to associate. This interpretation has yet to be confirmed as no clear evidence to support it has since been reported. This point was therefore further investigated here by studying phytochrome destruction as a function of temperature.

The destruction reaction has been examined in excised coleoptiles of etiolated maize seedlings following brief irradiation with monochromatic light, rather than during continuous irradiation, so as to be able to relate destruction to the initial levels of pelletability while avoiding cycling of phytochrome during destruction.

EXPERIMENTAL

Irradiation Procedure

Irradiation of excised coleoptiles was carried out at room temperature ($\sim 20^{\circ}\text{C}$) using the monochromator and interference filters described in Chapter 2. Irradiances at the wavelengths used were: 538nm: 1Wm^{-2} ; 578nm: 1.35Wm^{-2} ; 658nm: 0.25Wm^{-2} and 1.5Wm^{-2} ; 692nm: 1.5Wm^{-2} ; 717nm: 0.8Wm^{-2} and 1.5Wm^{-2} ; 730nm: 1.2Wm^{-2} . Five minutes of red light (658nm) at 1.5Wm^{-2} is sufficient to saturate both photo-

transformation and pelletability.

For short pulse (lms) irradiation with red light (650nm), the irradiation conditions were the same as those described in Chapter 5.

Incubation

Irradiated coleoptiles were kept in a temperature-controlled humidified dark chamber for dark reactions of phytochrome to proceed usually for 5-6 hours. Under these conditions, the treated tissues remain turgid for the entire experimental period.

Other conditions

Relevant safelight and Pfr measurement conditions are as stated in the text and captions to Figures and Tables. Phytochrome pelletability was determined using equation 3(8). Pfr measurements in 30KP fractions are derived from pellets resuspended in SEBM buffer. It follows that Figure 6.8 presented here is slightly different from that already published (Fig. 6, Fuad 1979).

RESULTS

Phytochrome destruction as a function of wavelength and dose of an inductive light pulse

To test the possible involvement of Pr and the relevance of pelletability in the dark destruction of phytochrome, the following experiments were carried out.

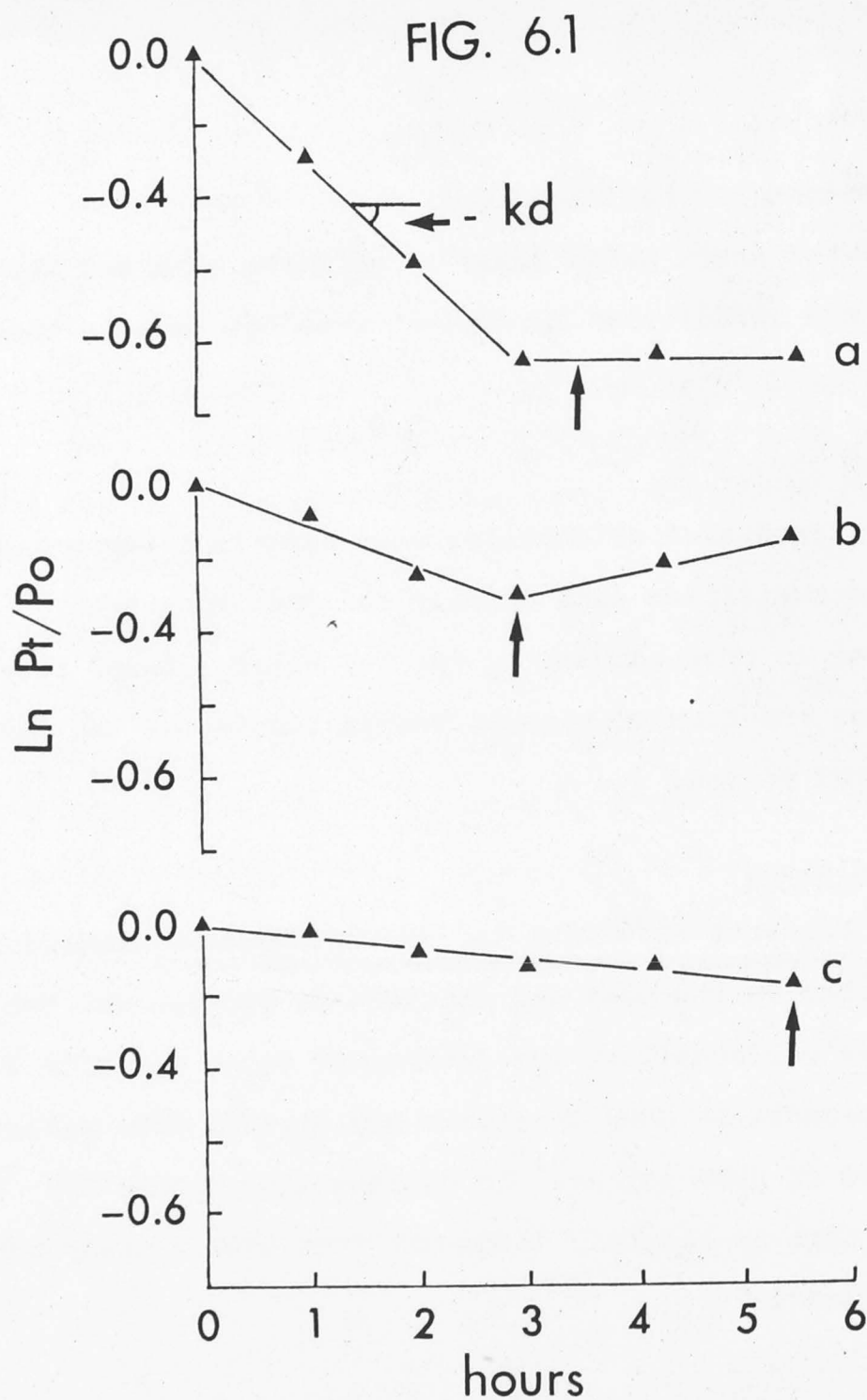


Fig. 6.1 Kinetics of phytochrome destruction *in vivo*. Following irradiation with the selected wavelength, coleoptiles were incubated in the dark at 30°C. P_t and P_o are phytochrome content at time t and time zero of incubation respectively. $\ln P_t/P_o$ is plotted as a function of duration of dark incubation.

Irradiations: a = 2 min. at $\lambda = 578\text{nm}$,
b = 2 min. at $\lambda = 538\text{nm}$,
c = 15 min. at $\lambda = 717\text{nm}$.

Coleoptiles were irradiated at a selected wavelength and then transferred immediately to incubate in the dark at 30°C. At zero time of incubation samples were analysed for initial pelletability, Pfr content and total phytochrome content. At scheduled times during incubation, samples were sacrificed to determine their phytochrome content by *in vivo* measurements. Typical curves for phytochrome destruction as assayed *in vivo* are shown in Figure 6.1. The reaction can be approximated to one of first order kinetics. The end of the reaction was usually characterised by the attainment of a steady state of total phytochrome content (Fig. 6.1a), probably reflecting a balance between destruction of phytochrome and resynthesis of Pr (see Boisard and Cordonnier 1976 for a list of references). This steady state can also be followed by an actual increase in the $\Delta(\Delta A)$ values (Fig. 6.1b). In some cases, however, (cf. Fig. 6.1c) the reaction did not reach a steady state situation at all.

From these curves, values for the rate of phytochrome destruction (k_d) and the extent of the destruction, i.e. the percentage of the total phytochrome that has disappeared by the end of the reaction, may be obtained. The lowest $\Delta(\Delta A)$ values (see arrows in Fig. 6.1) were taken to represent the extent of destruction before apparent resynthesis dominates. It is then possible to correlate the rate (k_d) and extent of destruction with the initial Pfr

content and with the initial level of pelletability as established immediately following the inductive irradiation. The last two parameters were found to vary with duration of irradiation, when subsaturating doses of light were used (see Chapter 4).

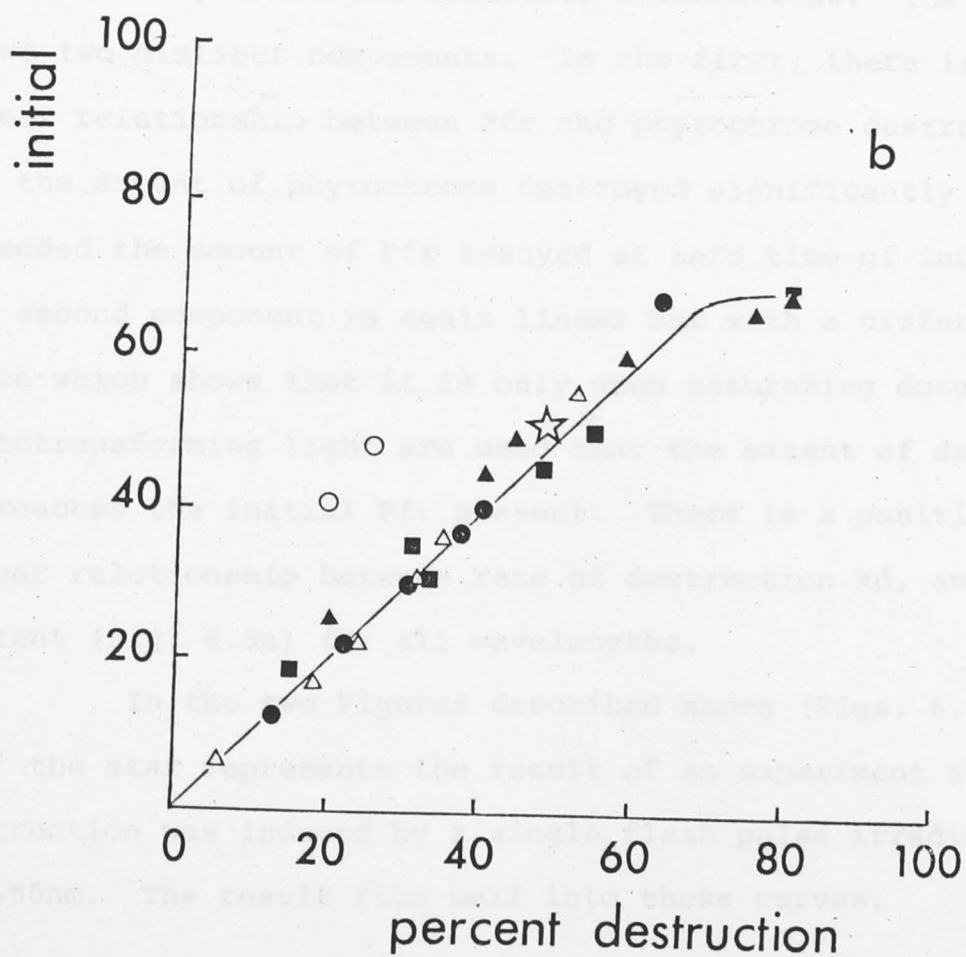
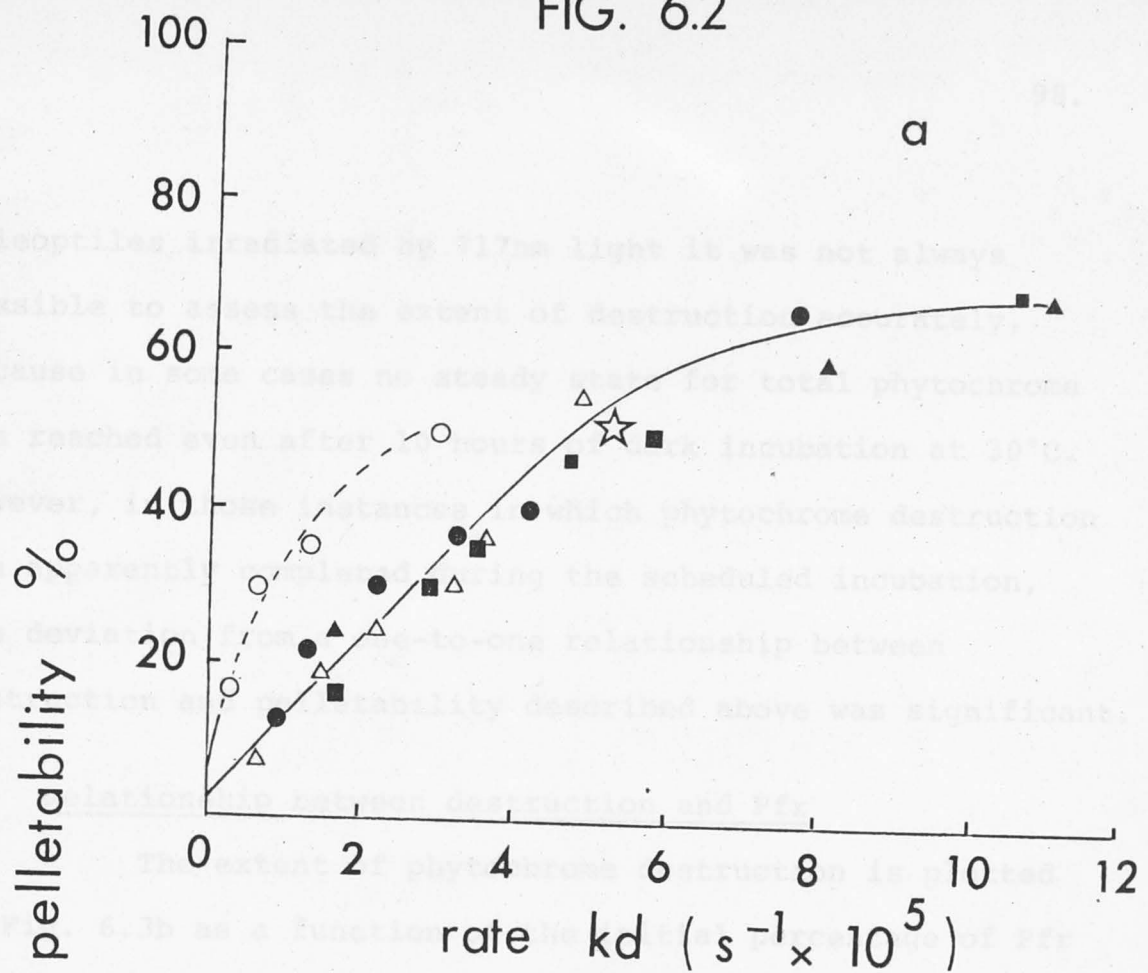
1. Relationship between destruction and pelletability

In Figs. 6.2a and 6.2b, k_d and extent of destruction are plotted as functions of the initial level of pelletability obtained with increasing light dose for five selected wavelengths. At all wavelengths tested there is a linear positive correlation between initial level of pelletability and rate or extent of destruction, at least up to an initial pelletability level of about 60 percent. The correlation deviates from linearity above 60 percent initial pelletability. For all wavelengths except 717nm, the slopes of the regression lines are indistinguishable, i.e. for the same initial pelletability value, phytochrome becomes destroyed at the same rate (k_d) irrespective of the wavelength used (538nm, 578nm, 658nm, 692nm) to induce destruction. In the case of $\lambda = 717\text{nm}$, for the same initial pelletability level, the rate of destruction is lower than for the other wavelengths tested. Also the slope (1.0) of the regression line between pelletability and destruction indicates that under 60 percent initial pelletability, for each phytochrome molecule bound at time zero, one molecule eventually becomes destroyed by the end of the scheduled dark incubation. In

Fig. 6.2 Correlation of phytochrome destruction with initial phytochrome pelletability.

Levels of phytochrome pelletability, which were established immediately following irradiation of coleoptiles with different doses of light of five selected wavelengths, are plotted as a function of the rate (a) and extent (b) of phytochrome destruction at 30°C in the dark. Selected wavelengths were 0 : 717nm, ● : 692nm, ■ : 658nm, ▲ : 578nm, △ : 538nm, ☆ : 650nm, flash-lamp irradiation. Pelletability was calculated using equation 3(8). The values shown are the means of at least 3 separate experiments.

FIG. 6.2



coleoptiles irradiated by 717nm light it was not always possible to assess the extent of destruction accurately, because in some cases no steady state for total phytochrome was reached even after 10 hours of dark incubation at 30°C. However, in those instances in which phytochrome destruction was apparently completed during the scheduled incubation, the deviation from a one-to-one relationship between destruction and pelletability described above was significant.

2. Relationship between destruction and Pfr

The extent of phytochrome destruction is plotted in Fig. 6.3b as a function of the initial percentage of Pfr established by different inductive irradiations. The curve shows two distinct components. In the first, there is a linear relationship between Pfr and phytochrome destruction, but the amount of phytochrome destroyed significantly exceeded the amount of Pfr assayed at zero time of incubation. The second component is again linear but with a different slope which shows that it is only when saturating doses of phototransforming light are used that the extent of destruction approaches the initial Pfr present. There is a positive linear relationship between rate of destruction k_d , and Pfr content (Fig. 6.3a) for all wavelengths.

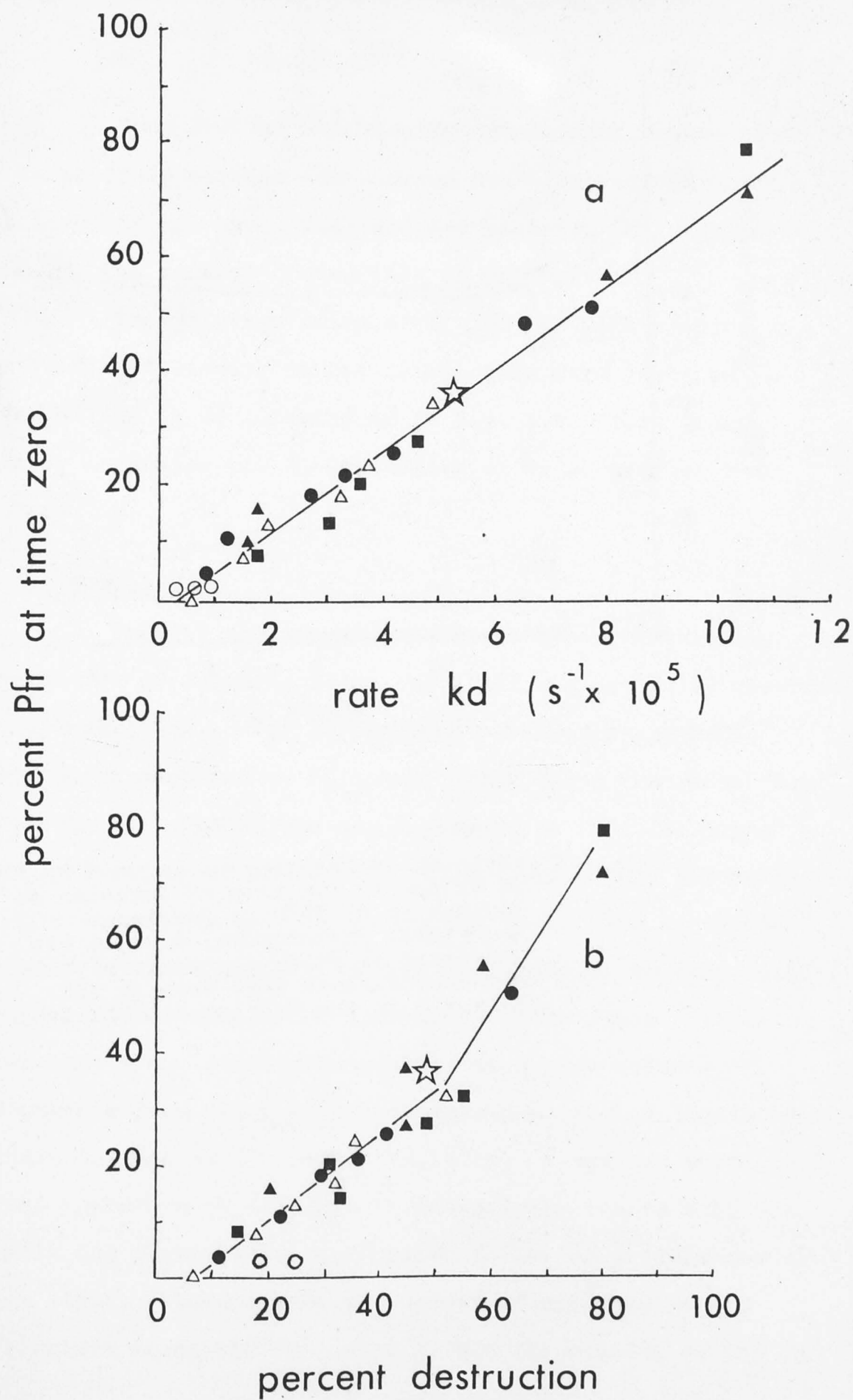
In the two Figures described above (Figs. 6.2 and 6.3) the star represents the result of an experiment in which destruction was induced by a single flash pulse irradiation at 650nm. The result fits well into these curves.

Fig. 6.3 Correlation of phytochrome destruction with Pfr content.

The Pfr content (expressed as percentage of total phytochrome), assayed *in vivo* immediately following irradiation by different doses of light of selected wavelengths, is plotted as a function of the rate (a) and extent (b) of the phytochrome destruction in the dark at 30°C. Wavelengths were represented by:
● : 692nm, ■ : 658nm, ▲ : 578nm,
△ : 538nm, ☆ : 650nm, flashlamp irradiation.

Pfr measurements were made using filter A in the Ratiospect and corrected using equation 3(5). SL: type B. Values shown are the means of at least 3 separate experiments.

FIG. 6.3



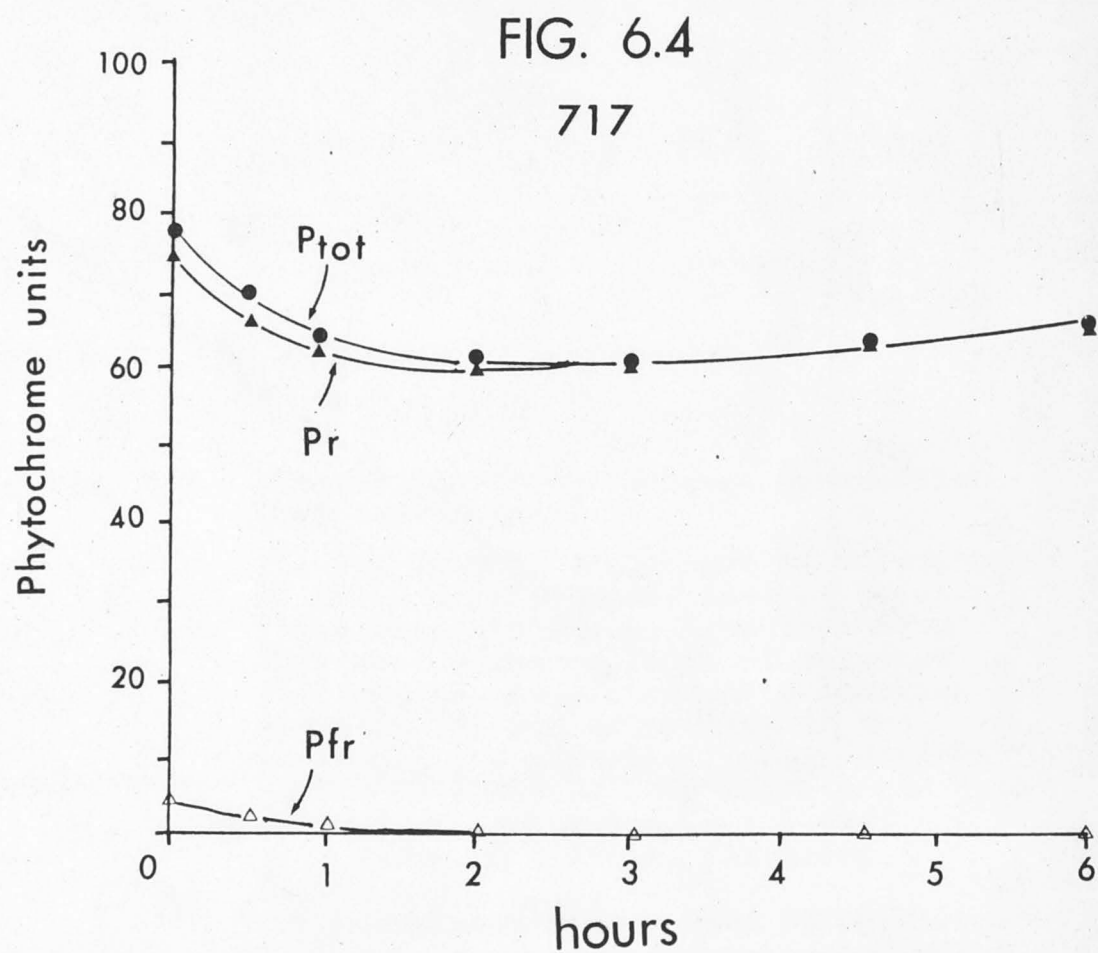


Fig. 6.4 Phytochrome destruction induced by light of 717nm, as function of duration of incubation at 30°C in darkness.
 ●—● total phytochrome, ▲ P_r, △ P_{fr}, expressed in $\Delta(\Delta A)$ phytochrome units. P_{fr} measurements were made *in vivo*, using filter B in the Ratiospect; SL: type C.

When $\lambda=717\text{nm}$ was used to induce dark destruction at least 20-26 percent destruction could be induced while the level of Pfr never exceeded 4-5 percent. This suggests that Pr can undergo destruction as well as Pfr.

The relative roles of Pr and Pfr with respect to destruction following 10 min irradiation with light of 717nm (1.6Wm^{-2}) is illustrated in Fig. 6.4. This figure clearly indicates the disappearance of Pr as well as that of Pfr.

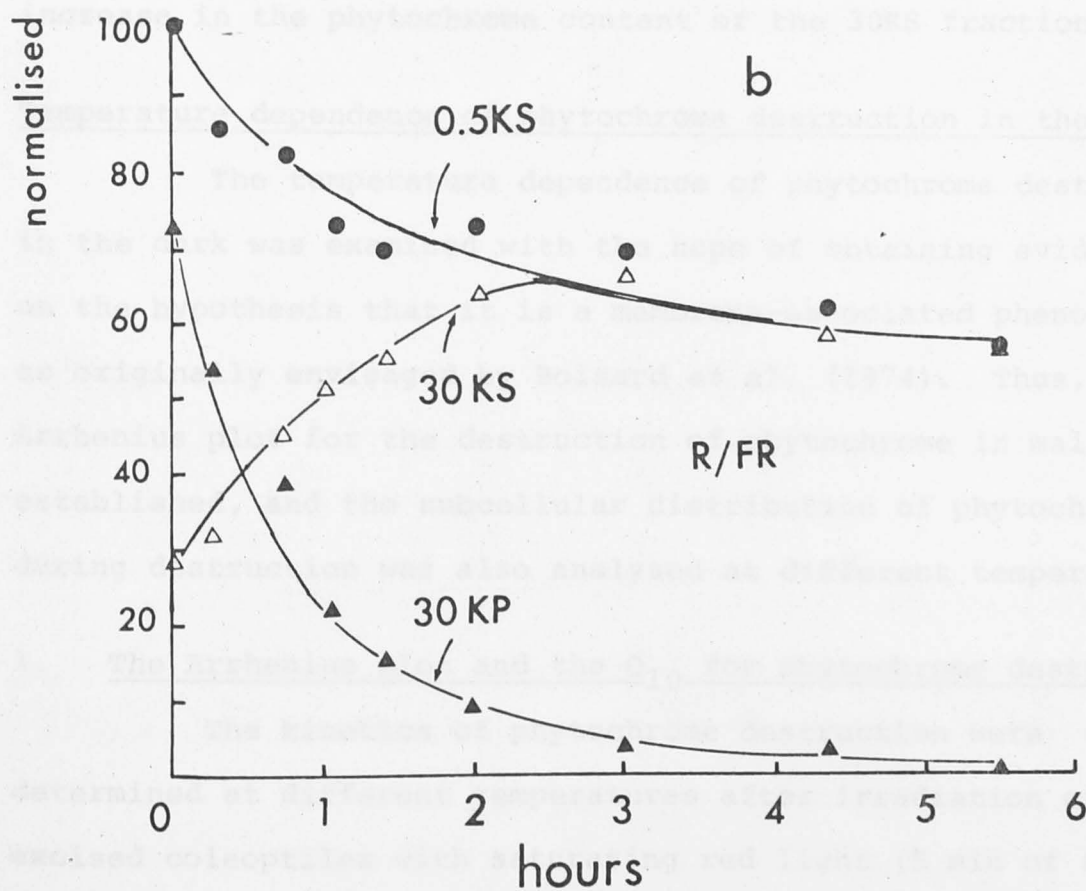
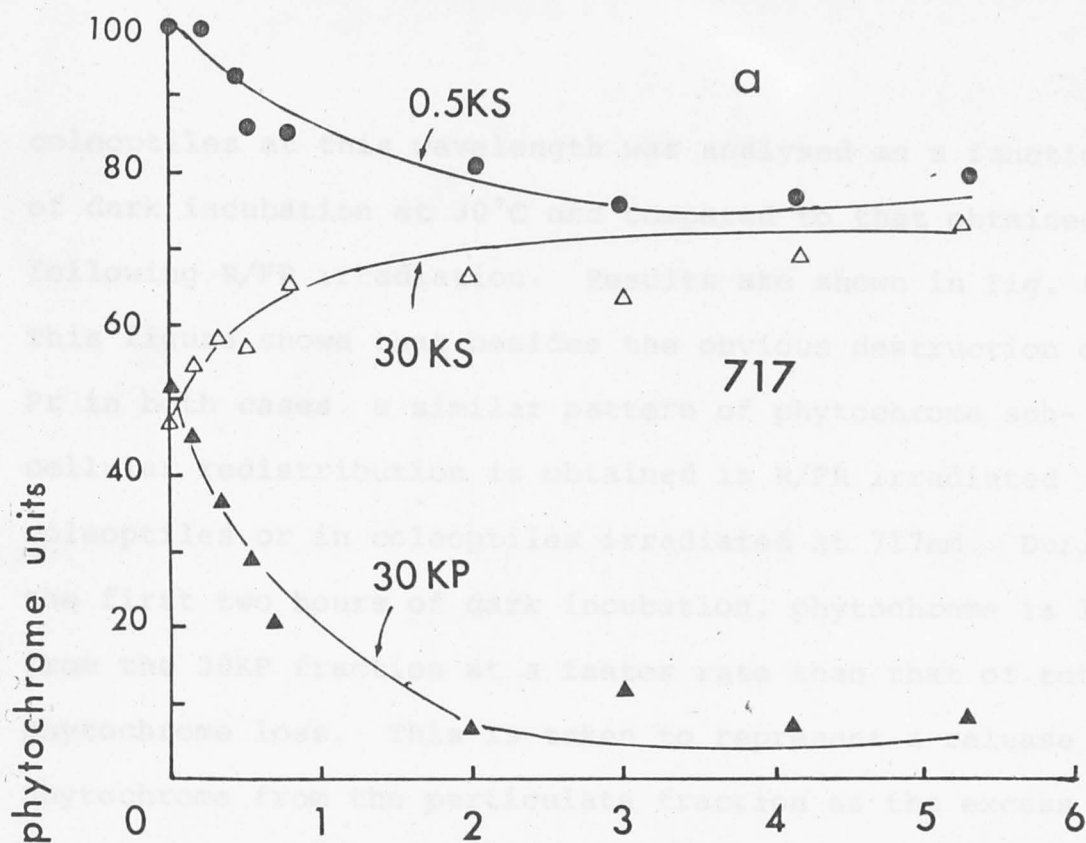
3. A comparison

Dooskin and Mancellini (1968) reported that the destruction of phytochrome by red light was not fully reversed by far-red light: R/FR irradiation enhanced phytochrome destruction compared to FR alone. They found that more than 20 percent of phytochrome was destroyed at 25°C following R/FR irradiation of *Avena* coleoptiles, while only 0-2 percent of phytochrome was present in the Pfr form. The situation reported by these authors is therefore similar to that found here, when destruction was induced by 717nm light. It is known that R/FR irradiation results in a slow release of phytochrome from the particulate fraction *in vivo* (Quail *et al.* 1973a; Boisard *et al.* 1974; Yu 1975b; Pratt and Marmé 1976). It was therefore of interest to investigate the fate of the soluble and particulate phytochrome following irradiation with 717nm light. The phytochrome content of the soluble and particulate fractions following 15 min irradiation of the

Fig. 6.5 Subcellular distribution of phytochrome during incubation at 30°C of coleoptiles irradiated with light of 717nm (a) or following red/far-red (b) irradiation.

●—● phytochrome content of the 0.5KS fraction,
Δ—Δ phytochrome content of the 30KS fraction,
▲—▲ phytochrome content of the 30KP fraction.
All values are normalised to phytochrome content of the 0.5KS fraction at time zero of incubation.
Phytochrome content in the 30KP fraction was calculated as $30KP = 0.5KS - 30KS$.

FIG. 6.5



coleoptiles at this wavelength was analysed as a function of dark incubation at 30°C and compared to that obtained following R/FR irradiation. Results are shown in Fig. 6.5. This figure shows that besides the obvious destruction of Pr in both cases, a similar pattern of phytochrome subcellular redistribution is obtained in R/FR irradiated coleoptiles or in coleoptiles irradiated at 717nm. During the first two hours of dark incubation, phytochrome is lost from the 30KP fraction at a faster rate than that of total phytochrome loss. This is taken to represent a release of phytochrome from the particulate fraction as the excess loss of phytochrome from the 30KP is compensated by an increase in the phytochrome content of the 30KS fraction.

Temperature dependence of phytochrome destruction in the dark

The temperature dependence of phytochrome destruction in the dark was examined with the hope of obtaining evidence on the hypothesis that it is a membrane-associated phenomenon as originally envisaged by Boisard *et al.* (1974). Thus, the Arrhenius plot for the destruction of phytochrome in maize was established, and the subcellular distribution of phytochrome during destruction was also analysed at different temperatures.

1. The Arrhenius plot and the Q_{10} for phytochrome destruction

The kinetics of phytochrome destruction were determined at different temperatures after irradiation of the excised coleoptiles with saturating red light (5 min of 658nm at

FIG. 6.6

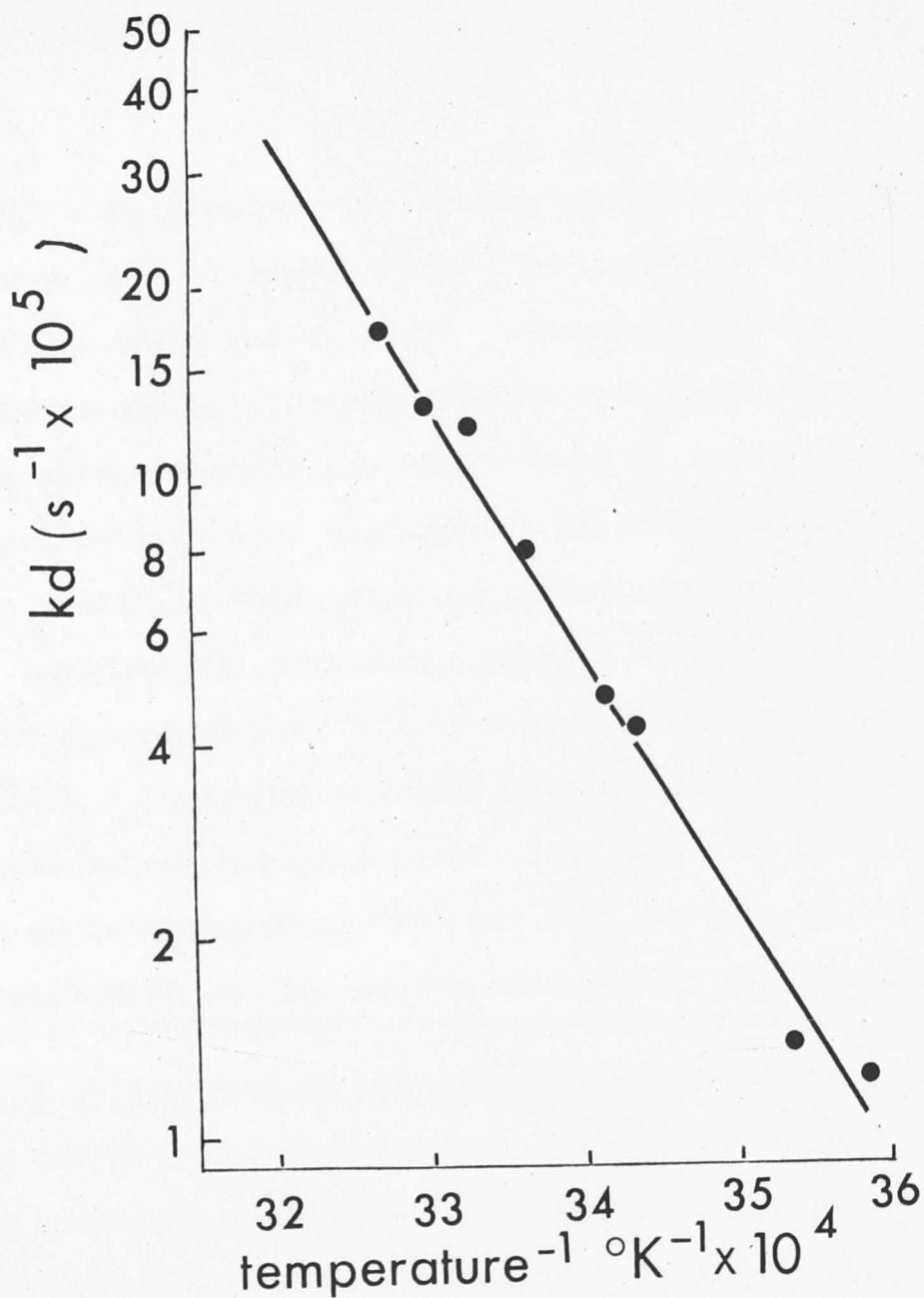


Fig. 6.6 Semilogarithmic plot of the rate of destruction k_d , as a function of the reciprocal of absolute temperature. Values of k_d were calculated for the destruction of phytochrome *in vivo* following 5 min. irradiation with red light (658nm) during 3.5 h dark incubation at different temperatures. Phytochrome content was measured in the 0.5KS fraction.

1.5Wm^{-2}). Phytochrome content was estimated from the $\Delta(\Delta A)$ readings in the 0.5KS fraction. As when assayed *in vivo*, the *in vitro* analysis showed apparent first order reactions above 0°C (no destruction was detectable at 0°C). The Arrhenius plot of k_d for the destruction reaction is illustrated in Fig. 6.6. Q_{10} values for temperature ranges between 10°C and 40°C are given in Table 6.1. All k_d values were calculated from the lines drawn according to the best fit from least square regression analysis of $\ln \text{Pt}/\text{Po}$ vs. time, taken over 3h 30min of dark incubation following red irradiation. As reported by Schäfer and Schmidt (1974) for *Cucurbita pepo*, the Arrhenius activation energy for the destruction reaction is found to be independent of temperature, as there is no discontinuity in the slope of the plot.

The Q_{10} values obtained are in agreement with those reported by Pratt and Briggs (1966) for the destruction of Pfr in intact maize coleoptiles: $Q_{10}^{14-24} = 3.5$, $Q_{10}^{24-34} = 2.7$.

2. Cellular distribution of phytochrome during dark incubation following 5 min irradiation with red light, as a function of temperature

The distribution of phytochrome in the soluble (30KS) and particulate fractions (30KP) was assayed at scheduled times during incubation of the irradiated coleoptiles at temperatures between 0°C and 40°C . Results are shown in Fig. 6.7.

Table 6.1 Q_{10} values for the destruction of phytochrome in maize coleoptiles.

Temperature °C	Kd $s^{-1} \times 10^5$	Q_{10}
10	1.35	
20	4.68	3.46
30	13.10	2.79
40	11.54	-0.88

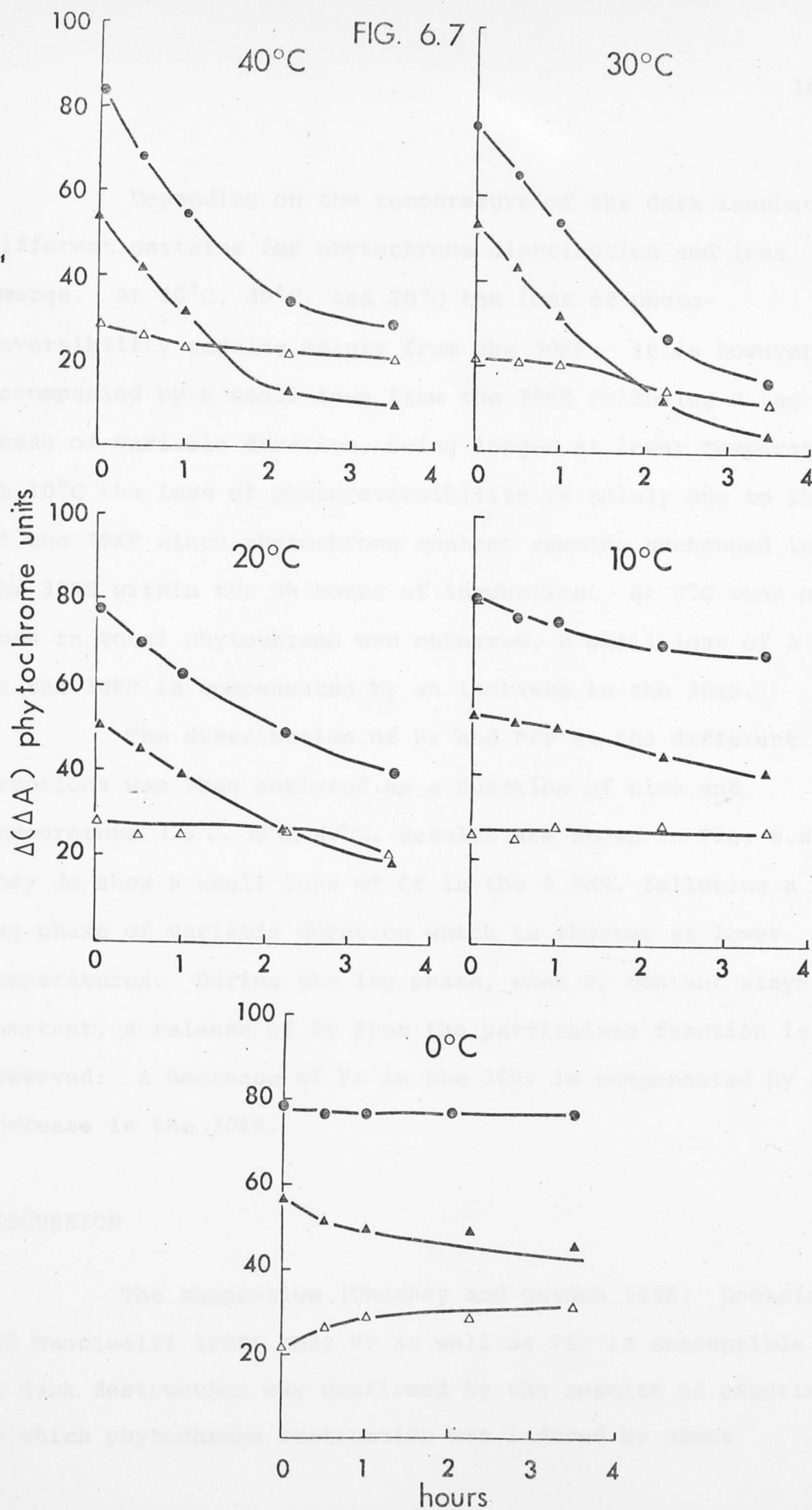
For experimental details see caption to Fig. 6.6.

Fig. 6.7

Subcellular distribution of phytochrome during incubation of red irradiated coleoptiles at different temperatures. Following 5 min. irradiation at 658nm, excised coleoptiles were incubated at a specified temperature in the dark. At scheduled times, samples were homogenised and fractionated for measurements of phytochrome distribution in the 0.5KS, 30KS and 30KP.

Phytochrome content in the 0.5KS (●—●), 30KS (△—△) and 30KP (▲—▲) is plotted as a function of the duration of dark incubation at the specified temperature. 30KP was either calculated from 0.5KS - 30KS or measured from the 30KP pellet resuspended in SEBM. Values represented are the means of at least 3 separate experiments.

FIG. 6.7



Depending on the temperature of the dark incubation, different patterns for phytochrome distribution and loss emerge. At 40°C, 30°C and 20°C the loss of photoreversibility results mainly from the 30KP: it is however accompanied by a small loss from the 30KS following a lag phase of variable duration, being longer at lower temperatures. At 10°C the loss of photoreversibility is solely due to that of the 30KP since phytochrome content remains unchanged in the 30KS within the 3½ hours of incubation. At 0°C when no loss in total phytochrome was observed, a small loss of $\Delta(\Delta A)$ in the 30KP is compensated by an increase in the 30KS.

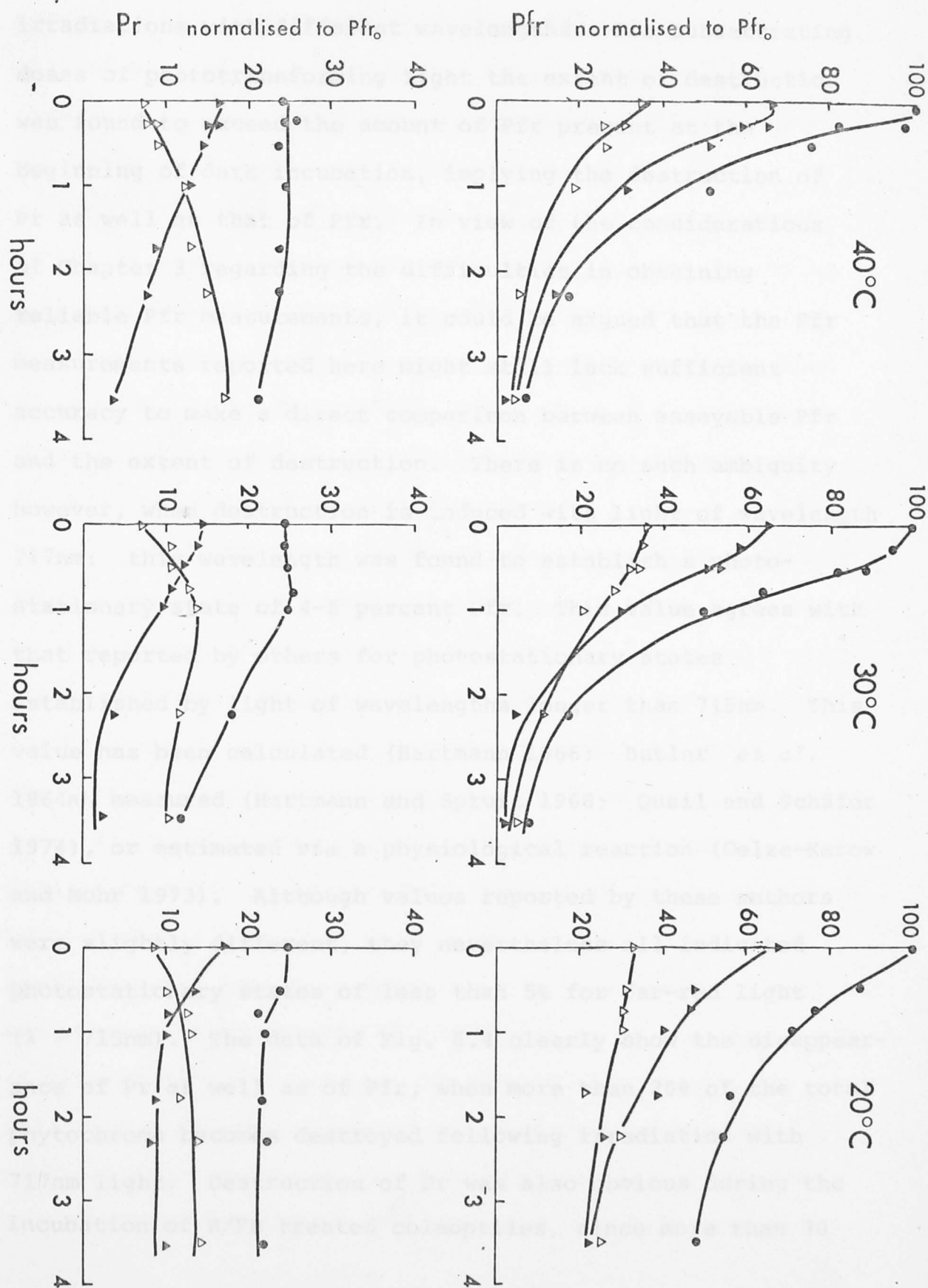
The distribution of Pr and Pfr in the different fractions was then analysed as a function of time and temperature (20°C, 30°C, 40°C). Results are shown in Fig. 6.8. They do show a small loss of Pr in the 0.5KS, following a lag phase of variable duration which is shorter at lower temperatures. During the lag phase, when Pr content stays constant, a release of Pr from the particulate fraction is observed: a decrease of Pr in the 30KP is compensated by an increase in the 30KS.

DISCUSSION

The suggestion (Chorney and Gordon 1966; Dooksin and Mancinelli 1968) that Pr as well as Pfr is susceptible to dark destruction was confirmed by the results of experiments in which phytochrome destruction was induced by short

Fig. 6.8 Pfr and Pr distribution in subcellular fraction of red irradiated coleoptiles during dark incubation at different temperatures. Percent of Pfr was assayed in the subcellular fractions at scheduled times during dark incubation at the specified temperature. Pr was calculated from $Pr = P_{tot} - P_{fr}$. The Pfr (upper frame) and Pr (lower frame) content of the subcellular fractions 0.5KS (●—●), 30KS (Δ—Δ), and 30KP (▲—▲) are plotted as a function of the duration of incubation in darkness at the specified temperature. All Pr and Pfr contents are normalised to Pfr content of the 0.5KS fraction at time zero of incubation. Pfr measurements were made with filter B in place in the Ratiospect. All 30KP fractions were resuspended in SEBM. SL: type C.

FIG. 6.8



irradiations with different wavelengths. For subsaturating doses of phototransforming light the extent of destruction was found to exceed the amount of Pfr present at the beginning of dark incubation, implying the destruction of Pr as well as that of Pfr. In view of the considerations of Chapter 3 regarding the difficulties in obtaining reliable Pfr measurements, it could be argued that the Pfr measurements reported here might still lack sufficient accuracy to make a direct comparison between assayable Pfr and the extent of destruction. There is no such ambiguity however, when destruction is induced with light of wavelength 717nm: this wavelength was found to establish a photostationary state of 4-5 percent Pfr. This value agrees with that reported by others for photostationary states established by light of wavelengths longer than 715nm. This value has been calculated (Hartmann 1966; Butler *et al.* 1964a), measured (Hartmann and Spruit 1968; Quail and Schäfer 1974), or estimated *via* a physiological reaction (Oelze-Karow and Mohr 1973). Although values reported by these authors were slightly different, they nevertheless all indicated photostationary states of less than 5% for far-red light ($\lambda > 715\text{nm}$). The data of Fig. 6.4 clearly show the disappearance of Pr as well as of Pfr, when more than 20% of the total phytochrome becomes destroyed following irradiation with 717nm light. Destruction of Pr was also obvious during the incubation of R/FR treated coleoptiles, since more than 30

percent of total phytochrome disappeared within 3h of incubation at 30°C. This result is in agreement with that obtained in *Avena* by Dooksin and Mancinelli (1968), which has since been confirmed in *Avena* by Mackenzie *et al.* (1978a). The results presented here differ from those of Mackenzie *et al.* in that the rate of destruction following R/FR irradiation found here is slower than that following red irradiation alone. The conclusion is inescapable that Pr, as well as Pfr, can undergo destruction and that there is no direct causal relationship between the presence of Pfr and the destruction of phytochrome.

For subsaturating doses of light the relationship between pelletability measured immediately after irradiation, and the extent or the rate of destruction, is positive and linear for each wavelength tested here. The data indicate a one-to-one relationship between pelletability and destruction for all wavelengths except 717nm when the initial level of pelletability is below 60 percent. Above this level, when percent Pfr is greater than percent pelletability, more phytochrome is destroyed than is pelletable. Under such conditions the extent of destruction approaches the amount of Pfr present in the tissue at the beginning of the incubation. In interpreting the results, one should bear in mind the following possible sources of inaccuracy in measurements. Possible inaccuracies in the determination of phytochrome pelletability and extent of destruction can only lead to an

underestimation of these quantities, due firstly to the apparent instability of the phytochrome-particle association *in vitro* (reported in Chapter 3), and secondly to the difficulty of deciding precisely when phytochrome destruction has been completed. This underestimation can not affect the conclusion already reached regarding the absence of an obligatory relationship between the amount of Pfr present following irradiation and the extent of destruction. Also, one can conclude that for each wavelength tested, the extent and the rate of destruction depend on the inductive light dose. The effect of the light dose on destruction does not seem to be mediated exclusively through the amount of Pfr measured following irradiation. Rather, it appears to correlate well with the amount of pelletable phytochrome induced by the irradiation, and it is only when percentage Pfr exceeds the level of pelletability that the extent of destruction appears to correlate with the amount of Pfr present. The effect of the light dosage could however be mediated by some other reaction of which we are unaware, but showing the same dose dependence as pelletability. I am therefore inclined to agree with Schäfer and co-authors (1975, 1976) concerning the existence of a fast binding reaction intervening between photoreaction and destruction, but differ from them however in maintaining that destruction is not exclusive to Pfr.

The question still remains whether or not the destruction reaction itself is a membrane associated phenomenon

as envisaged by Boisard *et al.* (1974) and Boisard and Cordonnier (1976).

Schäfer and Schmidt (1974) reported that in *Cucurbita pepo*, the Arrhenius activation energy for phytochrome destruction was constant over the range of temperature studied (0°C-40°C). I have extended this conclusion to the monocotyledon *Zea mays*. Although some authors have recently questioned the significance of Arrhenius plots in biological reactions (D.J. Bagnall, personal communication), breaks in the Arrhenius plot of a reaction have been interpreted to be indicative of a membrane-associated reaction (see Raison *et al.* 1971, 1977; Schäfer and Schmidt 1974 for references). The fact that no break in the Arrhenius plot of phytochrome destruction was found here does not provide any evidence for or against a membrane-associated reaction.

The study of phytochrome destruction as a function of temperature in maize suggests the existence of *in vivo* release of Pr from the potential particulate to the potential soluble fraction, during the dark incubation of irradiated coleoptiles. This is apparent when total Pr remains constant (i.e. during the lag phase preceding loss of Pr from the 0.5KS): Pr is lost from the particulate fraction and accumulates in the soluble fraction. As Pr destruction proceeds, Pr content in the 30KS remains essentially the same and loss of Pr is observed in the 30KP

only. It is possible therefore that Pr is released from the particulate fraction and subsequently destroyed in the soluble fraction, following a lag phase which is dependent on temperature. The same process might apply to Pfr, but if so it can not be observed clearly since following irradiation, destruction of Pfr occurs without a lag. Incidentally, the absence of a significant lag phase in the kinetics of total phytochrome destruction following 5 min red irradiation is in contrast with the results obtained by Schäfer *et al.* (1975), who found a lag phase of ~ 30 minutes. This may be due to different conditions used in growing maize (Kidd and Pratt 1973; Stone and Pratt 1978).

'Complex exchanges of Pr and Pfr' between fractions have been suggested for *Cucurbita pepo* by Boisard and Cordonnier (1976) although, as discussed by the authors, the nature of the pelletability in this material has been the subject of criticism (Quail 1975a, b). Recently, Cordonnier *et al.* (1978) have analysed computer simulations of various possible models that they described for phytochrome destruction. Although these models do not allow for Pr destruction, they concluded that a straight destruction of soluble Pfr without transition by the particulate fraction cannot be accounted for from a comparison of the simulated kinetics with experimental data obtained.

In view of possible exchanges of phytochrome between the soluble and the particular fraction *in vivo*, and assuming

that the capacity of the receptor to bind phytochrome is limiting (see Chapters 4 and 5), one may speculate that in the case where percentage Pfr exceeds percentage pelletability, if binding sites become vacated in the destruction process (Boisard *et al.* 1974), hitherto unbound molecules might interact with them in the dark and thereafter become susceptible to destruction. Such a mechanism would reconcile the concept of the existence of a binding reaction step prior to destruction with the experimental observation that in the cases of $\lambda = 658\text{nm}$, and $\lambda = 578\text{nm}$, more destruction than initial pelletability is obtained (Fig. 6.2).

In the case where phytochrome destruction was induced by R/FR or 717nm irradiation, less destruction than initial pelletability was observed. In these cases phytochrome was found to be released from the particulate fraction during the first two hours of incubation. This may indicate that the destruction of Pr actually occurs at the binding site: thus a release of Pr from the particulate fraction (i.e. the binding site) would prevent its destruction. Results obtained in the study of phytochrome destruction as a function of temperature suggested the opposite mechanism. An alternative interpretation therefore may be that besides undergoing a fast binding reaction prior to destruction, phytochrome must remain associated with the particulate binding partner for some time before it becomes susceptible

to destruction. Premature release of Pr from the particulate fraction would therefore result in reduced susceptibility to destruction.

To summarise, the results of experiments described in this chapter indicate destruction of Pr, they also support the hypothesis of Schäfer that a fast binding reaction step precedes destruction. No evidence however was found to support the hypothesis that phytochrome destruction is a membrane associated phenomenon. It seems that prior to the destruction of Pr, its release from a particulate binding partner may be involved. In the case of Pfr destruction, however, the problem remains unsolved. An immunocytochemical approach (cf. Mackenzie *et al.* 1978b) may be able to offer an answer.

CHAPTER 7

GENERAL DISCUSSION

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The conclusions already drawn from the data presented in the previous chapters are briefly summarized below. Chapter 3 demonstrated a demonstration of the involvement of phytochrome in the regulation of the photoperiodic response. This chapter also demonstrated that phytochrome is involved in the regulation of the photoperiodic response. In Chapter 4 it was demonstrated that phytochrome is involved in the regulation of the photoperiodic response. The establishment of the action spectrum also supports the hypothesis that the induction of phytochrome photoperiodicity in maize is a genuine phytochrome-mediated intracellular process. While red light was most effective in producing high levels of photoperiodicity in short irradiations, under continuous irradiation higher photoperiodicity levels were obtained in PR and blue regions than under red. PR and Pfr were both found to be involved in phytochrome photoperiodicity and phytochrome destruction. The conclusion was reached from flash experiments in Chapter 5 that phytochrome can become photoperiodic without going through the Pfr state, and the existence of intermediates of photoperiodicity was also indicated by a pulse technique.

CHAPTER 7

GENERAL DISCUSSION

The conclusions already drawn from the data presented in the previous chapters are briefly summarised below. Chapter 3 demonstrated a number of difficulties involved in reliable measurements of phytochrome and Pfr content, and the determination of phytochrome pelletability in maize. This Chapter also illustrated that phytochrome *in vitro* may behave differently according to its origin. In Chapter 4 phytochrome was demonstrated to be the photo-receptor for its own pelletability when pelletability was induced by short irradiations. The establishment of the action spectrum also supports the hypothesis that the induction of phytochrome pelletability in maize is a genuine phytochrome-mediated intracellular process. While red light was most effective in producing high levels of pelletability in short irradiations, under continuous irradiation higher pelletability levels were obtained in FR and blue regions than under red. Pr and Pfr were both found to be involved in phytochrome pelletability and phytochrome destruction. The conclusion was reached from flash experiments in Chapter 5 that phytochrome can become pelletable without going through the Pfr state, and the existence of intermediates of photo-transformation was made evident using a double flash technique

to induce phototransformation. Finally, in Chapter 6, while Pr destruction was demonstrated, phytochrome destruction appeared to correlate well with initial levels of pelletability attained following the inductive irradiation.

The determination of the light induced phytochrome pelletability necessarily implies disruption of the tissue, and the presence of a divalent cation (known to cause aggregation of subcellular material) in the medium is necessary to observe it. It could be held therefore that the resulting enhanced pelletability may not reflect an intracellular light induced association between phytochrome (P) and a reaction partner (X), which becomes pelletable upon tissue fractionation, but instead that the capacity of phytochrome to associate with X is potentiated *in vivo*, and that the actual association occurs during homogenisation.

Pratt and Marmé (1976) and Quail (1978a) have shown that the induction of phytochrome pelletability develops in the dark following red irradiation. Hence, as already inferred by the establishment of the action spectrum, at least one or several dark reactions following absorption of light by phytochrome and leading to enhanced phytochrome pelletability, are necessarily occurring *in vivo*. This is also supported by the fact that the induction of phytochrome pelletability in response to red light can be substantially reduced or slowed down in the presence of metabolic

inhibitors (Yu 1977, 1978b; Quail and Briggs 1978). This suggests an energy dependent reaction step in the induction of phytochrome pelletability, possibly involving an ATP-dependent modification of Pfr or X (Quail and Briggs 1978, Yu 1978b).

Arguments in favour of the hypothesis that Mg^{++} retains a phytochrome particle association existing *in vivo*, rather than that it induces an artifactual association with particulate material *in vitro* are gathered below.

Pratt and Marmé (1976) observed that after extraction of red irradiated tissue in Mg^{++} -free buffer, addition of Mg^{++} , which aggregates subcellular material, does not yield any enhanced pelletability of phytochrome.

Quail (1978a) showed that rapid post-homogenisation addition of Mg^{++} to homogenates extracted in Mg^{++} -free buffer can sustain the enhanced pelletability, but with rapidly decreasing efficiency. From these observations (made on *Avena*), Pratt (1978) concluded that 'if Mg^{++} induces a coprecipitation of phytochrome with other subcellular material, this coprecipitation must occur at or near the moment of extraction in response to a Pfr-induced change in the tissue'.

In Chapter 3 another argument was presented in favour of the preservation *in vitro* by Mg^{++} of a state of the phytochrome molecule existing *in vivo*. The spectral characteristics of phytochrome *in vivo* following R/FR irradiation were found to be similar to those of pelleted

phytochrome when it was resuspended in the presence of Mg^{++} , but different from those of pelleted phytochrome resuspended in the absence of Mg^{++} . These data are consistent with the hypotheses that (i) either the molecular environment of phytochrome has not changed upon extraction in the presence of Mg^{++} , or (ii) that *in vivo*, phytochrome has undergone a conformational change affecting its spectral characteristics, which can only be retained *in vitro* in the presence of Mg^{++} .

The first alternative would argue strongly for preservation of an association *in vivo* between P and X which appears to involve an ionic bond (Yu and Carter 1976a; Quail 1978b). The second alternative would only argue for an *in vivo* interaction between P and X if the postulated conformational change results from such an interaction with X, rather than being merely a consequence of phototransformation (see Chapter 5, model 3) and this possibility remains open.

It is of interest to note here that some differences in the molecular properties of native Pr and pelleted Pr (different behaviour in gel exclusion chromatography and different electrophoretic mobility), have been observed which could be interpreted in terms of changes in conformation of the protein (Yu and Carter 1976a, b; Grombein and Rüdiger 1976; Boeshore and Pratt 1978). Boeshore and Pratt suggested that, although the difference observed probably arises *in vitro*, it cannot be excluded that an undetected modification of the protein *in vivo* might also result in different reactivity of phytochrome *in vitro*.

The role of Mg^{++} in the *in vivo* enhanced pelletability phenomenon could be interpreted in the following manner.

In vivo, Mg^{++} stabilises an interaction between X and P. The interaction need not be the formation of a complex between X and P, however in the absence of such a complex, a close proximity between X and P is implied which makes an ionic bridging by Mg^{++} between X and P possible. This Mg^{++} bridge or preservation of complex would be lost upon homogenisation in the absence of Mg^{++} either a) by dilution of the intracellular Mg^{++} or chelation by EDTA present in the buffer, and/or b) by the inevitable disorganisation and increased perturbation of the system during homogenisation. There are indications that only a low concentration of Mg^{++} would be needed to preserve this association *in vitro* (Quail 1978b, Table 3.1).

Incidentally, it may be possible to test this scheme by performing post-homogenisation addition of Mg^{++} experiments (cf. Quail 1978a), when homogenisation would be carried out in a buffer of high viscosity. It would be hoped that in such conditions the molecular perturbation resulting from homogenisation of the tissue might be reduced, and therefore that rupture of the postulated interaction might be slowed. Hence the enhanced pelletability could be rescued by the addition of Mg^{++} for a longer time following homogenisation.

The scheme above therefore favours the induction by light of an interaction between P and X, occurring *in vivo*, and which is maintained *in vitro* in the presence of Mg^{++} . Arguments against the alternative hypothesis, that a change in the phytochrome molecule induced by light *in vivo* merely results in a 'stickiness' of the molecule *in vitro* to subcellular components in the presence of Mg^{++} (Lehman and Schäfer 1978), come from the study of Yu and co-workers. These authors have obviated the requirement for a divalent cation in the extraction medium to maintain pelletability, by treating the tissues with crosslinking reagents before homogenisation (Yu 1975a, b, c; Yu and Carter 1976c; Yu *et al.* 1976). Yu (1978a) pointed out that a successful crosslinking by bifunctional reagents requires a correct and stable orientation and disposition of the two proteins involved. Therefore, following irradiation, phytochrome and X must be positioned in such a way that they can be crosslinked with a bifunctional reagent such as glutaraldehyde. The data obtained by Yu and co-workers therefore indicate either:

- i) that phytochrome *in vivo* has actually moved from a more distant position in relation to X before irradiation to a closer position following irradiation, making crosslinking possible; or
- ii) that phytochrome *in vivo* is very close to the partner X, and a change of configuration of phytochrome *in vivo* following irradiation makes crosslinking possible.

The data of Mackenzie *et al.* (1975) would support the first interpretation since they showed by immunocytochemistry that in *Avena* coleoptile parenchyma cells, phytochrome (Pr) has a diffuse distribution; following irradiation with red light however, phytochrome becomes discretely localised or sequestered. Mackenzie *et al.* (1978b) also pointed out that 'there is a close correspondence between immunocytochemically observed redistribution of phytochrome and its existence in an at least potentially pelletable state induced by *in vivo* irradiation..... . Thus, *in vivo* induced phytochrome pelletability and the immunocytochemically observed redistribution may be two assays for the same process'.

The various studies mentioned above of the sub-cellular localisation of phytochrome in maize and oats following irradiation, are therefore all consistent with the following interpretation: the light enhanced association of phytochrome with X, observed following fractionation of irradiated tissues, is the result of an intracellular change of P and/or X induced by light, necessarily coupled with a close proximity of phytochrome to X *in vivo*, at least following irradiation. The nature of this change is as yet uncertain, nevertheless it results in enhanced phytochrome pelletability. In what follows the phrase 'induction of phytochrome pelletability' is used to refer to this change. There is good evidence that X is a membrane component (Marmé *et al.* 1974).

Hence the close proximity mentioned above has the potential to result in an intracellular interaction between phytochrome and membranes, and such an interaction may be of particular relevance in the modulation of membrane properties by phytochrome which was proposed by Hendricks and Borthwick (1967).

We now have to deal with the question of whether or not the *in vivo* induced pelletability is of any relevance in the induction of other phytochrome-mediated responses.

The relationship between phytochrome pelletability and phytochrome destruction has been established (Chapter 6). The results suggested that the induction of phytochrome pelletability may indeed be a necessary step prior to the destruction of phytochrome. Hence for the destruction reaction, which is another intracellular phytochrome-mediated response, the induction of phytochrome pelletability appears to be a relevant physiological reaction.

Is phytochrome destruction itself an important reaction in the mode of action of phytochrome? If so, the conclusion just reached could be used to partly demonstrate the relevance of the induction of phytochrome pelletability in the mode of action of phytochrome.

While some phytochrome-mediated responses do not appear to correlate with phytochrome destruction (e.g. coleoptile elongation in *Avena*, Dooksin and Mancinelli 1968), some authors have reported good correlations between physiological

responses and phytochrome destruction (see Chapter 6). Smith and Attridge (1970) proposed that 'phytochrome decay may be an integral part of the mechanism of action of phytochrome'. Hartmann (1966) and Schäfer (1975c, d) also attribute an essential role to phytochrome destruction in their interpretation of HIR. Thus it is clear that phytochrome destruction could be implicated in some phytochrome-mediated physiological responses. In turn therefore, the induction of phytochrome pelletability appears to be a relevant physiological reaction in the mode of action of phytochrome.

Ideally, to fully demonstrate the relevance of the induction of pelletability, one should investigate a large number of phytochrome-mediated responses in relation to induction of phytochrome pelletability in the same system. A complementary approach would be to find an *in vitro* system in which phytochrome could be photo-induced to associate with a defined subcellular component and, as a result, control photoreversible responses in this fraction; such a system would also be very useful in the search for a receptor X (Marmé 1978). Roux and Yguerabide (1973) found that phytochrome can spontaneously enter and induce photoreversible conductance changes in black lipid membranes. While the *in vitro* induced association of phytochrome with ribonucleoprotein reported for *Cucurbita* is believed to be artifactual and of no biological significance (Quail 1975c; Pratt 1978), reports have already

shown that phytochrome can be photo-induced to bind to purified mitochondria preparations from *Avena* (Georgevich *et al.* 1977) and to isolated organelles from barley leaves (Smith *et al.* 1978). This binding is consistent with a non-artifactual association of soluble phytochrome to organelle membranes, at least in the latter case.

In this thesis, data were presented which demonstrated that Pr as well as Pfr becomes pelletable following irradiation. Hence the interaction between P and X is not restricted to Pfr. It also appeared from flash experiments that this interaction can take place between X and phytochrome which has not been through the Pfr state, and that intermediates of phototransformation may be involved in the pelletability phenomenon. Thus it is clear that other forms than Pfr can interact with X. In the hypothesis that the induction of phytochrome pelletability is of relevance to the control of a morphogenic response by phytochrome, it becomes necessary to envisage that other forms of phytochrome than Pfr may play an active role in the mode of action of phytochrome. This possibility became apparent in the present study as it was shown that, like Pfr, Pr is involved in the destruction of phytochrome.

In the hypothesis that phytochrome pelletability may have an important relationship to the control of a response, irrespective of the chromophoric configuration (Pr or Pfr) of the molecule, variability in the times of escape from reversibility could be explained in the following manner.

1. Some responses may be irreversibly triggered once the interaction between P and X, leading to enhanced phytochrome pelletability, has taken place. Such responses would then be expected to have very fast escape times, like the induction of pelletability (2 sec at 25°C, Quail and Briggs 1978). Reversal in these cases would require that Pfr is converted to Pr before it has the chance of interacting with X.
2. Other responses reversible by FR within 3-30 min may require phytochrome to remain in the pelletable state before a reaction leading to the response is fully triggered. As suggested above, if only the maintenance of the pelletable state of the molecule at a minimum level and for a critical duration is important irrespective of the chromophoric state of the molecule, then far-red light can suppress the response. This is because R/FR can cause a more rapid loss of the pelletable state than does red light alone, at least at low temperatures (Chapter 3).

The possibility suggested above that other forms of phytochrome than Pfr might be involved in the mode of action of phytochrome have been envisaged by other workers.

Hillman (1965) proposed that, in the destruction process, Pfr may decay to a spectrophotometrically undetectable active form. Smith and Attridge (1970) also considered this as a possible way of interpreting a good correlation found between phytochrome destruction and the rate of increase in PAL activity.

Hopkins (1971) observed that the control by phytochrome over elongation of *Avena* coleoptiles is a function of the changing ratio of Pr (or conversely Pfr) to total phytochrome, rather than absolute levels of Pfr. He proposed a more functional role of Pr in photomorphogenesis, classically attributed to Pfr alone.

Hillman (1967) suggested that the existence of intermediates of phototransformation, even if short lived, raises the possibility that some of them might be physiologically active. In the present study, it was also suggested that intermediates of phototransformation may be involved in the pelletability phenomenon. Experiments with double flash irradiations suggested the existence of at least two intermediates in the Pr to Pfr pathway, which decay with half-lives of approximately 8ms and 26ms. Briggs and Fork (1969) observed the accumulation (under conditions of pigment cycling) of Meta Rb, which decayed within a few seconds. Rüdiger (1978) found an intermediate with the spectral properties of Meta Ra which relaxes to Pr within minutes or hours. As a result of his investigation, Rüdiger (1978) suggested the existence of two kinds of equilibrium for phytochrome:

(i) A true photoequilibrium is that established during irradiation and represents an equilibrium between Pr, Pfr and all intermediates of phototransformation.

(ii) A thermodynamic equilibrium on the other hand is reached in darkness, after relaxation of all intermediates to either Pr or Pfr.

In explaining the mode of action of phytochrome, it may be valuable to consider these situations separately. A static mode of action operating via Pfr (or Pfr and Pr) may be considered when thermodynamic equilibrium is reached; a dynamic mode of action for phytochrome, operating possibly via intermediates of phototransformation, may be considered as long as phytochrome is photoexcited. These ideas are not new. It has been proposed that HIR (equivalent to dynamic) and induction responses (equivalent to static) do not operate via the same effector molecule (Schopfer and Mohr 1972; Mohr 1972; Schäfer *et al.* 1975c, d), and Jose and Vince-Prue (1978) have gathered arguments which support these views. While it is agreed amongst these authors that, in the induction reaction, phytochrome acts in the ground state, the irradiance dependency of HIR has resulted in controversial interpretations. Hartmann (1966) considered, but rejected, intermediates of phototransformation as effectors for HIR. Mohr (1972) and Schopfer and Mohr (1972) considered that phytochrome acts via an excited state of Pfr. Hartmann and Unser (1973) rejected this interpretation and attributed the irradiance dependence of HIR to a greater activity of nascent Pfr than of old Pfr. This is not unlike Schäfer's interpretation by which the effector for HIR is a transient state of

Pfr (PfrX) prior to transition to PfrX', the effector in induction reactions (Schäfer 1973c, d). None of these hypotheses however have received positive demonstration to date. For example, it is not known what the biochemical differences between 'old' and 'nascent' Pfr or between X and X' may be. As already discussed before (Chapter 5), the transition PfrX to PfrX' invoked to explain enhanced Pr pelletability is based on a theory of cooperativity in binding, which has not been demonstrated to apply to the phytochrome-binding site system.

As an alternative to these hypotheses, I would like to propose here that, in a dynamic situation, it may be the actual transformation of intermediates of photo-transformation into others and/or into Pfr and Pr which may partly be responsible for phytochrome action. It is known that in vision, complete bleaching of rhodopsin to opsin + all-trans-retinal is too slow to account for the brief delay of visual excitation following light absorption. In invertebrates, it is proposed that the transformation of Metarhodopsin I478 to Metarhodopsin II380 is the most likely reaction step responsible for visual excitation; it is also the reaction which involves the major conformation changes of the pigment (Ostroy 1977 for a review). In the photo-transformation of phytochrome, a major conformational change probably occurs from the relaxations of Meta Rb and Meta Fb,

and these are the slowest reactions in the Pr to Pfr to Pr cycle (Kendrick and Spruit 1977). It can therefore be envisaged that these transformations may be of physiological importance. For instance in the membrane hypothesis it can be postulated that, under dynamic conditions, continuous light-driven transformation of intermediates involving important conformational changes may lead to changes in electrical fields in the molecular environment. This could lead to modulation of membrane properties. This modulation could occur whether phytochrome is (i) an integral component of a membrane, (ii) is externally bound to a membrane, or (iii) is in close proximity to a membrane (Hill 1967). The number of intermediates that undergo transformation in a given time will be a function of phytochrome levels, its molecular environment, light intensity, wavelength, temperature etc. This kind of approach to phytochrome action thus has the potential to explain the irradiance dependency of HIRs, it also has the capacity to account for perception of light quality and duration of light periods, as envisaged by Jose and Vince-Prue (1978).

In induction-reversion responses, the suppression of a red-induced response by far-red light could similarly be related to the conformational changes that take place following far-red irradiation, i.e. during transformation of Pfr to Pr. These may be more important in the suppression of some responses than the simple removal of 'active' Pfr

from the system. It could be postulated that the transformation of Pfr to Pr (as opposed to removal of Pfr alone), may trigger a further reaction which has a feedback inhibitory effect on the development of the red-induced morphogenic response. This feedback could be exerted on an early or late reaction step involved in the expression of the response, hence this mechanism has the potential to account for both slow and fast escape from reversibility. This scheme may also offer a solution to some phytochrome paradoxes, like the 'pea paradox' (Hillman 1965, see Chapter 1), because it has the advantage that only little Pfr need be present at the time of far-red irradiation for the transformation Pfr to Pr to have an effect.

In the hypothesis advanced above, that phytochrome action is not solely dependent on activity of Pfr, it is obvious that 'phytochrome paradoxes' cease to exist, since they arise from the assumption that Pfr is the only active form of phytochrome, and from a lack of correlation between the amount of spectrophotometrically assayable Pfr and the degree of a physiological response in a number of cases (see Chapter 1).

Other approaches to explain the phytochrome paradoxes can be envisaged however.

- 1) It is possible that phytochrome paradoxes are partly due to difficulties of obtaining reliable Pfr measurements. These difficulties were demonstrated in Chapter 3. Of particular relevance here are the problems

associated with far-red transmission of safelights and a small red component in the far-red actinic beam in Ratiospects.

While it is often stated by some authors that the safelights in use in their laboratory do not cause conversion of Pr to Pfr, mention is rarely made of the effect of safelights on the Pfr to Pr conversion. If safelights used do contain a far-red component, such as those described in Fig. 2.1a, b, then appreciable loss of spectrophotometrically assayable Pfr should be expected upon exposure of Pfr containing material to safelights, commensurate with the duration of exposure.

Pfr measurements at low Pfr levels are rendered difficult by a small red component in the actinic irradiation used in the spectrophotometric assay. While some authors are well aware of this problem (Hillman 1965, 1966; Briggs and Chon 1966), some others report negative $\Delta(\Delta A)$ values without explanation. Hence it is difficult to evaluate how widespread this problem may be.

Calculations of Pfr content *in vivo* are necessarily based on *in vitro* determinations of $[Pr]_{\infty}^{\lambda}$ and $[Pfr]_{\infty}^{\lambda}$ values (see Chapter 3). Until these values can be established to be the same *in vivo* in various tissues, all measurements of Pfr *in vivo* or in crude homogenates, obtained by spectrophotometric assay, are potentially inaccurate. The results presented in this thesis have been interpreted with this reservation in mind.

2) The second aspect of the problem is related to the fact that phytochrome measurements *in vivo* or in crude extracts are necessarily average measurements of all the phytochrome situated in the path of the measuring and actinic beams of the Ratiospect. This may include phytochrome contained in various organs, tissues, cells or molecular environments. Kendrick and Spruit (1973c) suggested that 'it appears likely that the phytochrome controlling a specific physiological response is present only in one particular environment'. This suggestion is in good agreement with the interpretation of Briggs and Chon (1966) that 'only a small portion of the coleoptile phytochrome might be involved in the observed physiological response'. Hence the notion of bulk and active phytochrome seems justified. I would like to point out here however, that the concept of bulk phytochrome should not be equated with inactive phytochrome. It is likely that that portion of phytochrome which may appear inactive in the control of any response under investigation may be active in the control of other responses. Phytochrome controlling different responses is also likely to be situated at different loci in the seedling, and it is also known that phytochrome controlling a response in one organ can be situated in another (Oelze-Karow and Mohr 1974). At low light intensity the transformation of phytochrome will be very much dependent on its location within the seedling relative to the light source. Some responses may therefore be induced by the photo-transformation of the fraction of the total phytochrome which is involved in the control of a response. The photo-

transformation of this fraction could be facilitated by its location as compared to the rest of the phytochrome, while the phytochrome as a whole would not have absorbed sufficient light to undergo phototransformation.

To conclude, the induction of phytochrome pelletability appears to reflect an important intracellular interaction between phytochrome and a subcellular component. In my view, any physiological relevance of this interaction is most easily understood in terms of an interaction between phytochrome and a cellular membrane. The diversity of morphogenic responses could be explained on the hypothesis that the membraneous receptor is located at various loci in the cell. The loci of the receptors would be the specificity factor in membrane action. For instance, phytochrome action on leaflet movements could be explained if the receptor X is situated on the vacuole membrane, or the plasmalemma, regulating ion fluxes. In the hypothesis of regulation by phytochrome of gene expression, the receptor could be located on the nuclear membrane, and phytochrome interaction could lead to either direct interaction with the genome or changes in the export of RNA etc. Alternatively, it could be located on the endoplasmic reticulum, leading to interaction with ribosomes.

It should be stressed here that the suggestion in this discussion of physiological activity for other forms than Pfr, should not be taken as a denial of activity for Pfr.

It is felt here however, that the rigid concept of Pfr as the only active form of phytochrome should be made more flexible to include other active phytochrome species.

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PUBLICATIONS

Publications resulting from the work carried out in the course of tenure of my ANU Ph.D. Scholarship are listed below.

- YU R.; FUAD N. and CARR D.J. 1976 In vivo phototransformation of phytochrome in relation to its binding to a particulate fraction of maize coleoptiles. *Plant Cell Physiol.* 17: 1131-1139.
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